THE UNIVERSITY
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Predicting outcome in Crohn's disease

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THE UNIVERSITY
of EDINBURGH

Thesis to be submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2020

Contents

Abstract

Crohn's disease is a chronic immune-mediated disease which can cause inflammation in the gastrointestinal tract anywhere between mouth and anus. It is now understood to be the result of a complex interplay between genetic, microbial and environmental factors. The detail of these interactions remains an important focus of research. Clinicians and patients are faced with an increasing range of therapeutic options for Crohn's. Key decision points include first presentation to gastrointestinal services, assessment of risk of disease progression, escalation to biologic therapy, and withdrawal of immunomodulator or biologic therapies. Biomarkers are needed that help stratify patients at these times to facilitate shared decision-making. In this thesis, I have brought together seven of the first-author papers published from the work conducted during my period of study.

I first investigated the associations between *NOD2*, the strongest genetic association for Crohn's disease, and the faecal microbiome. *NOD2* encodes a bacterial pattern recognition receptor, and so disease-associated genetic variants might be expected to lead to an alteration in the microbiota. Although I demonstrated clear associations between the presence of inactive Crohn's and changes in the faecal microbial composition, I was unable to show an impact of *NOD2* genotype in either patients with Crohn's or in volunteers without gastrointestinal disease.

Calprotectin is the dominant protein in the cytosol of neutrophils. Its measurement in faecal samples has become well-established as a tool for non-invasive assessment of intestinal inflammation. I have investigated its use here for both diagnosis and prognosis in Crohn's disease. In the diagnosis paper, I have assessed the diagnostic accuracy in of faecal calprotectin in a large cohort of patients referred to secondary care with lower

gastrointestinal symptoms. I demonstrated a high negative predictive value, with very few false negatives even after three years of follow-up. In patients with established Crohn's disease, I have then gone onto show that higher concentrations of faecal calprotectin, independent of symptoms, are associated with a greater risk of progressing to stricturing or penetrating disease, hospitalisation or resectional surgery.

All of the therapies used in Crohn's disease carry long-term risks including an elevated risk of infection and certain cancers. For this reason, among others, clinicians and patients must regularly assess the risk-benefit of continued therapy versus withdrawal of medication. I conducted two national, multicentre studies to better understand the outcomes of treatment withdrawal; one for thiopurines, and the other for anti-tumour necrosis factor alpha (anti-TNF), two of the most widely used classes of maintenance medication for Crohn's disease. I demonstrated a relapse rate of 39% at two years following withdrawal of thiopurines for Crohn's disease, and for 56% two years following anti-TNF withdrawal. I identified factors predictive of an increased risk of relapse, and for anti-TNF performed a systematic review and meta-analysis of all of the available published data.

Anti-TNF therapies were the first targeted biologic therapy licensed for Crohn's disease and they remain an important tool for the induction and maintenance of remission. Their introduction has been transformative for many patients, but secondary loss of response is common. In the final paper included in this thesis, I present the results from PANTS, a 120-site study of infliximab and adalimumab in 1,610 patients with Crohn's disease. I have demonstrated the importance of good early drug concentrations for outcome, and explored the relationship between patient factors, drug and anti-drug antibody concentrations and longer term outcomes.

As part of the PANTS study, we have collected longitudinal biological samples, and building on the clinical data presented in this thesis, I have described some of the early downstream analyses of genetic associations with anti-drug antibody formation, as well as plans for analyses of DNA methylation, transcription, serum protein concentrations, immunoglobulin G glycosylation and variation in the microbiome.

This thesis brings together work spanning a breadth of translational and clinical research into Crohn's disease. I have contributed to better understanding of pathogenesis, and to prediction of outcome at key decision points within the clinical disease course. These projects, particularly the PANTS study, provide a strong foundation for further research to better understand this important disease.

Lay summary

Crohn's disease is one of the two main types of inflammatory bowel disease. It most commonly causes inflammation of the small and large bowel. We currently think that Crohn's disease results from a combination of the genes we inherit from our parents, environmental factors such as smoking and diet and the types of bacteria or bugs that live in our bowels.

We have made much progress in understanding Crohn's disease over the past twenty years, but there remain many unanswered questions. In this thesis, I have explored the relationship between one of the inherited risk factors for Crohn's disease and the types of bacteria in the bowel. I have also sought to better understand how we can diagnose Crohn's disease, and how we can help doctors and patients make better treatment decisions.

In the first part of my thesis, I describe a study where we collected stool samples from patients with Crohn's disease and from healthy volunteers. I used modern molecular techniques to compare the types of bacteria found in the stool between people who had different variants of a gene, NOD2, associated with Crohn's disease. Although I was able to see differences between the Crohn's disease patients and the healthy volunteers, the genetic variant did not make a difference.

In the rest of my thesis, I have looked at predicting outcome at various critical points: diagnosis, risk of disease worsening, starting a first targeted biologic therapy and withdrawal of therapy. At diagnosis, I have analysed data on the use of faecal calprotectin, a stool test that can help detect inflammation in the bowel. I have then also looked at how

useful this test is to predict whether a patient with Crohn's disease will have worsening of their disease and require admission to hospital or surgery.

Treatments for Crohn's disease are often taken for a long time, and both doctors and patients want to know when and if they can consider stopping them. Using data from patients around the UK, I have described in one chapter the chances of relapse of disease following stopping azathioprine or mercaptopurine, and things that can help predict the risk of relapse. In another chapter, I have done the same for the anti-TNF drugs infliximab and adalimumab.

Finally, I have analysed data from over 1600 patients from around the UK who were started on infliximab or adalimumab for the first time. I have demonstrated the importance of getting good levels of the drug and identified ways of predicting whether patients will respond.

Declaration of Originality

I declare that this thesis has been written solely by me, other than where indicated below, and has not been submitted, in whole or in part, for another degree or qualification.

Each of the individual chapters has been published as a full paper as indicated in the list of publications and in the header/footer of the papers. I was the first author on each paper and have detailed my contributions in the introduction to each paper. The overall introduction and discussion, as well as the specific introductions/summaries for each chapter, are my work exclusively and have not been published elsewhere.

Signed: .....

Nicholas A Kennedy

Date: .....

Acknowledgements

This thesis represents seven years of part-time work and has given me the opportunity to work with a several leading researchers in clinical and translational inflammatory bowel disease. I am grateful to Professor Jack Satsangi for offering me the chance to work as part of his group in Edinburgh and supporting me through the various sub-projects that have arisen during this time. I have enjoyed working alongside the rest of Prof Satsangi's team as it has evolved over the years including Elaine Nimmo, Alex Adams, Paul Henderson, Nicholas Ventham, Rahul Kalla, Nidhi Sharma, Kate O'Leary, Hazel Drummond and Colette McColl. I am grateful to David Wilson and Sarah Howie who along with Elaine Nimmo and Jack Satsangi have provided guidance for my thesis.

I am also thankful for the support and opportunities I have had working with Dr Charlie Lees at the Western General Hospital. Dr Lees and I co-ordinated several of the multisite projects detailed in this thesis. I am also grateful for contributions from the other clinical consultants at the Western General Hospital and the many research fellows and junior doctors who worked on these projects alongside Dr Lees and me, particularly Gareth Jones and Nikolas Plevris.

The work on the gut microbiota detailed here were made possible by collaboration with Professor Georgina Hold who has also provided invaluable external mentorship during this period. I would like to thank her research assistant Susan Berry who helped supervise me in lab techniques while working in Aberdeen and Alan Walker who contributed to the 16S analyses.

The final part of my thesis centres on the PANTS study, a prospective study of anti-tumour necrosis factor alpha therapy in Crohn's disease that involved over 1600 patients across 120

UK sites. I am grateful to Professor Tariq Ahmad, Claire Bewshea, James Goodhand and the research team in Exeter for all their support with my work on this project.

Finally, I would like to thank all of the patients who have contributed samples and data to the projects encapsulated in this thesis.

List of publications and awards arising from this thesis

Prizes and awards

Investigator Initiated Study Award from the European Crohn's and Colitis Organisation for oral presentation on PANTS study (2018)

John Scrimgeour Prize from NHS Lothian for work on anti-TNF withdrawal (2014)

Anne Ferguson Prize from Scottish Society of Gastroenterology for presentations on faecal calprotectin and anti-TNF withdrawal (2014)

Publications in this thesis

Chapter 4. **Kennedy NA**, Walker AW, Berry SH, Duncan SH, Farquarson FM, Louis P, et al. The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. PLoS One 2014;9:e88982.

Chapter 5. **Kennedy NA**, Lamb CA, Berry SH, Walker AW, Mansfield J, Parkes M, et al. The Impact of NOD2 Variants on Fecal Microbiota in Crohn's Disease and Controls Without Gastrointestinal Disease. Inflamm Bowel Dis 2018;24:583–92.

Chapter 6. **Kennedy NA**, Clark A, Walkden A, Chang JCW, Fascí-Spurio F, Muscat M, et al. Clinical utility and diagnostic accuracy of faecal calprotectin for IBD at first presentation to gastroenterology services in adults aged 16-50 years. J Crohns Colitis 2015;9:41–9.

Chapter 7. **Kennedy NA**, Jones GR, Plevris N, Patenden R, Arnott ID, Lees CW. Association Between Level of Fecal Calprotectin and Progression of Crohn's Disease. Clin Gastroenterol Hepatol. 2019 Feb 14. pii: S1542-3565(19)30180-6

Chapter 8. **Kennedy NA**, Kalla R, Warner B, Gambles CJ, Musy R, Reynolds S, et al. Thiopurine withdrawal during sustained clinical remission in inflammatory bowel disease: relapse and recapture rates, with predictive factors in 237 patients. Aliment Pharmacol Ther 2014;40:1313–23.

Chapter 9. **Kennedy NA**, Warner B, Johnston EL, Flanders L, Hendy P, Ding NS, et al. Relapse after withdrawal from anti-TNF therapy for inflammatory bowel disease: an observational study, plus systematic review and meta-analysis. Aliment Pharmacol Ther 2016;43:910–23.

Chapter 10. **Kennedy NA**, Heap GA, Green HD, Hamilton B, Bewshea C, Walker GJ, et al. Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn's disease: a prospective, multicentre, cohort study. lancet Gastroenterol Hepatol. 2019 Feb 26 (epub ahead of print). doi: 10.1016/S2468-1253(19)30012-3

The first six of these publications (chapters 4 – 9) have been published under a creative commons licence which provides permission for their inclusion. At the time of original submission, chapter 7 has been published online, but final proofs of the typeset article were not available. The text of the and figures of the accepted, peer-reviewed article have therefore been reproduced here. Copyright for the seventh publication has been assigned to the publisher, but I have retained author rights that include permission to include the publication in a thesis.

The Lancet's policy on inclusion of published articles within a thesis can be found at http://www.thelancet.com/pb/assets/raw/Lancet/authors/lancet-jpa.pdf. It includes the following (emphasis in bold italics added):

References are included for each chapter within the individual publications. References for the text outside of the main body of chapters have been included separately at the end of the thesis.

Publications arising from my doctoral studies

In addition to the publications included in the main body of this thesis, work on the following co-authored publications was conducted while working towards my PhD:

1. Nimmo ER, Prendergast JG, Aldhous MC, **Kennedy NA**, Henderson P, Drummond HE, et al. Genome-wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway. Inflamm Bowel Dis 2012;18:889–99.
2. Henderson P, Casey A, Lawrence SJ, **Kennedy NA**, Kingstone K, Rogers P, et al. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease. Am J Gastroenterol 2012;107:941–9.
3. **Kennedy NA**, Clark DN, Bauer J, Crowe AM, Knight AD, Nicholls RJ, et al. Nationwide linkage analysis in Scotland to assess mortality following hospital admission for Crohn's disease: 1998-2000. Aliment Pharmacol Ther 2012;35:142–53.
4. **Kennedy NA**, Rhatigan E, Arnott IDR, Noble CL, Shand AG, Satsangi J, et al. A trial of mercaptopurine is a safe strategy in patients with inflammatory bowel disease intolerant to azathioprine: an observational study, systematic review and meta-analysis. Aliment Pharmacol Ther 2013;38:1255–66.
5. Ventham NT, **Kennedy NA**, Duffy A, Clark DN, Crowe AM, Knight AD, et al. Nationwide linkage analysis in Scotland-Has mortality following hospital admission for Crohn's disease changed in the early 21st century? J Crohns Colitis 2014; Epub ahead of print Sep 26, 2014. Available from doi:10.1016/j.crohns.2014.09.002.
6. Adams AT, **Kennedy NA**, Hansen R, Ventham NT, O'Leary KR, Drummond HE, et al. Two-stage genome-wide methylation profiling in childhood-onset Crohn's Disease

implicates epigenetic alterations at the VMP1/MIR21 and HLA loci. Inflamm Bowel Dis 2014;20:1784–93.

7. Ventham NT, **Kennedy NA**, Duffy A, Clark DN, Crowe AM, Knight AD, et al. Comparison of mortality following hospitalisation for ulcerative colitis in Scotland between 1998-2000 and 2007-2009. Aliment Pharmacol Ther 2014;39:1387–97.
8. Henderson P, **Kennedy NA**, Van Limbergen JE, Cameron FL, Satsangi J, Russell RK, et al. Serum C-reactive Protein and CRP Genotype in Pediatric Inflammatory Bowel Disease. Inflamm Bowel Dis 2015;21:596–605.
9. Trbojević Akmačić I, Ventham NT, Theodoratou E, Vučković F, **Kennedy NA**, Krištić J, et al. Inflammatory bowel disease associates with proinflammatory potential of the immunoglobulin G glycome. Inflamm Bowel Dis 2015;21:1237–47.
10. Ventham NT, Gardner RA, **Kennedy NA**, Shubhakar A, Kalla R, Nimmo ER, et al. Changes to serum sample tube and processing methodology does not cause inter-individual variation in automated whole serum N-Glycan profiling in health and disease. PLoS One 2015;10:e0123028.
11. Walker RM, Christoforou AN, McCartney DL, Morris SW, **Kennedy NA**, Morten P, et al. DNA methylation in a Scottish family multiply affected by bipolar disorder and major depressive disorder. Clin Epigenetics 2016;8:5.
12. Cleynen I, Boucher G, Jostins L, Schumm LPP, Zeissig S, Ahmad T, … **Kennedy NA** et al. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: A genetic association study. Lancet 2016;387:156–67.
13. Kalla R, **Kennedy NA**, Ventham NT, Boyapati RK, Adams AT, Nimmo ER, et al. Serum Calprotectin: A Novel Diagnostic and Prognostic Marker in Inflammatory Bowel Diseases. Am J Gastroenterol 2016;111:1796–805.

14. Mowat C, Arnott I, Cahill A, Smith M, Ahmad T, Subramanian S, et al. (**Kennedy NA** penultimate author). Mercaptopurine versus placebo to prevent recurrence of Crohn's disease after surgical resection (TOPPIC): a multicentre, double-blind, randomised controlled trial. lancet Gastroenterol Hepatol 2016;1:273–82.
15. Ventham NT, **Kennedy NA**, Adams AT, Kalla R, Heath S, O'Leary KR, et al. Integrative epigenome-wide analysis demonstrates that DNA methylation may mediate genetic risk in inflammatory bowel disease. Nat Commun 2016;7:13507.
16. Satsangi J, **Kennedy NA**, Mowat C, Arnott I, Keerie C, Lewis S, et al. A randomised, double-blind, parallel-group trial to assess mercaptopurine versus placebo to prevent or delay recurrence of Crohn's disease following surgical resection (TOPPIC). NIHR Journals Libr 2017; Epub ahead of print 2017. Available from doi:10.3310/eme04040.
17. Robertson AR, **Kennedy NA**, Robertson JA, Church NI, Noble CL. Colonoscopy quality with Entonox®vs intravenous conscious sedation: 18608 colonoscopy retrospective study. World J Gastrointest Endosc 2017;9:471–9.
18. Heerasing N, Thompson B, Hendy P, Heap GA, Walker G, Bethune R, **Kennedy NA**, Ahmad T, Goodhand JR. Exclusive enteral nutrition provides an effective bridge to safer interval elective surgery for adults with Crohn's disease. Aliment Pharmacol Ther 2017;45:660–9.
19. de Lange KM, Moutsianas L, Lee JC, Lamb CA, Luo Y, **Kennedy NA**, et al. Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. Nat Genet 2017;49:256–61.
20. Luo Y, De Lange KM, Jostins L, Moutsianas L, Randall J, **Kennedy NA**, et al. Exploring the genetic architecture of inflammatory bowel disease by whole-genome sequencing identifies association at ADCY7. Nat Genet 2017;49:186–92.

21. Walker GJ, Moore L, Heerasing N, Hendy P, Perry MH, Mcdonald TJ, …, **Kennedy NA**, Goodhand JR, Ahmad T. Faecal calprotectin effectively excludes inflammatory bowel disease in 789 symptomatic young adults with/without alarm symptoms: A prospective UK primary care cohort study. Aliment Pharmacol Ther 2018;47:1103–16.
22. Šimurina M, de Haan N, Vučković F, **Kennedy NA**, Štambuk J, Falck D, et al. Glycosylation of Immunoglobulin G Associates With Clinical Features of Inflammatory Bowel Diseases. Gastroenterology 2018;154:1320–1333.e10.
23. Klasić M, Markulin D, Vojta A, Samaržija I, Biruš I, Dobrinić P,… **Kennedy NA** et al. Promoter methylation of the MGAT3 and BACH2 genes correlates with the composition of the immunoglobulin G glycome in inflammatory bowel disease. 2018;10:75.
24. Jones GR, Fascì-Spurio F, **Kennedy NA**, Plevris N, Jenkinson P, Lyons M, et al. Faecal Calprotectin and Magnetic Resonance Enterography in Ileal Crohn's Disease: Correlations Between Disease Activity and Long-Term Follow-Up. J Crohns Colitis. 2019 Mar 30;13(4):442–50.
25. Walker GJ, Harrison JW, Heap GA, Voskuil MD, Andersen V, Anderson CA,… **Kennedy NA,** Ahmad T. Association of Genetic Variants in NUDT15 With Thiopurine-Induced Myelosuppression in Patients With Inflammatory Bowel Disease. Jama. 2019;321(8):773–85.

Reviews

26. Ventham NT, **Kennedy NA**, Nimmo ER, Satsangi J. Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. Gastroenterology 2013;145:293–308.

27. Jones GR, **Kennedy NA**, Lees CW, Arnott ID, Satsangi J. Systematic review: The use of thiopurines or anti-TNF in post-operative Crohn's disease maintenance--progress and prospects. Aliment Pharmacol Ther 2014;39:1253–65.
28. Theodoratou E, Campbell H, Ventham NT, Kolarich D, Pučić-Baković M, Zoldoš V, et al. The role of glycosylation in IBD. Nat Rev Gastroenterol Hepatol 2014;11:588–600.
29. Kalla R, Ventham N., **Kennedy NA**., Quintana JF, Nimmo ER, Buck AH, et al. MicroRNAs: new players in IBD. Gut 2015;64:504–13.
30. Torres J, Boyapati RK, **Kennedy NA**, Louis E, Colombel J-F, Satsangi J. Systematic Review of Effects of Withdrawal of Immunomodulators or Biologic Agents from Patients with Inflammatory Bowel Disease. Gastroenterology 2015;149:1716–30.
31. Ventham NT, Kalla R, **Kennedy NA**, Satsangi J, Arnott ID. Predicting outcomes in acute severe ulcerative colitis. Expert Rev Gastroenterol Hepatol 2015;9:405–15.

Editorials

32. **Kennedy NA**, Satsangi J. Editorial: Early corticosteroids in ulcerative colitis. Aliment Pharmacol Ther 2014;40:727.
33. Heerasing N, **Kennedy NA**. Interaction Between NOD2 and Smoking in the Pathogenesis of Crohn's Disease. EBioMedicine 2017;21. Epub ahead of print Jun 2017. Available from doi:10.1016/j.ebiom.2017.06.016.
34. **Kennedy NA**, Goodhand JR, Rampton DS. Editorial: which iron preparation for patients with IBD? Aliment Pharmacol Ther 2017;46:194–5.

Letters to the Editor

35. **Kennedy N**, Bauer J, Clark D, Crowe A, Knight A, Nicholls J, et al. Mortality in patients hospitalised with Crohn's disease: authors' reply. Aliment Pharmacol Ther 2012;35:397–8.

36. Jones GR, **Kennedy NA**, Lees CW, Arnott ID, Satsangi J. Letter: faecal calprotectin and lactoferrin - accurate biomarkers in post-operative Crohn's disease - authors' reply. Letter: biologic therapies are effective for prevention of post-operative Crohn's disease recurrence - authors' reply. Aliment Pharmacol Ther 2014;40:323.
37. **Kennedy NA**, Lees CW. Letter: Azathioprine-induced pancreatitis and subsequent tolerance of mercaptopurine - Authors' reply. Aliment Pharmacol Ther 2014;39:440–1.
38. **Kennedy NA**, Keerie C, Mowat C, Satsangi J. A plea for TDM-based optimisation for treatment of Crohn's disease – Authors' reply. Lancet Gastroenterol Hepatol 2017;2:81.

Listed as a contributor

39. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119–24.
40. Lee JC, Espéli M, Anderson CA, Linterman MA, Pocock JM, Williams NJ, et al. Human SNP links differential outcomes in inflammatory and infectious disease to a FOXO3-regulated pathway. Cell 2013;155:57–69.
41. Chen G-B, Lee SH, Brion M-JA, Montgomery GW, Wray NR, Radford-Smith GL, et al. Estimation and partitioning of (co)heritability of inflammatory bowel disease from GWAS and immunochip data. Hum Mol Genet 2014;23:4710–20.
42. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet 2015;47:979–86.
43. Goyette P, Boucher G, Mallon D, Ellinghaus E, Jostins L, Huang H, et al. High-density mapping of the MHC identifies a shared role for HLA-DRB1*01:03 in inflammatory

bowel diseases and heterozygous advantage in ulcerative colitis. Nat Genet 2015;47:172–9.

44. Coleman C, Quinn EM, Ryan AW, Conroy J, Trimble V, Mahmud N, et al. Common polygenic variation in coeliac disease and confirmation of ZNF335 and NIFA as disease susceptibility loci. Eur J Hum Genet 2016;24:291–7.
45. Rivas MAMA, Graham D, Sulem P, Stevens C, Desch ANN, Goyette P, et al. A protein-truncating R179X variant in RNF186 confers protection against ulcerative colitis. Nat Commun 2016;7:12342.Bodea CA, Neale BM, Ripke S, Barclay M, Peyrin-Biroulet L, Chamaillard M, et al. A Method to Exploit the Structure of Genetic Ancestry Space to Enhance Case-Control Studies. Am J Hum Genet 2016;98:857–68.
46. Schwerd T, Bryant RV, Pandey S, Capitani M, Meran L, Cazier J-B, et al. NOX1 loss-of-function genetic variants in patients with inflammatory bowel disease. Mucosal Immunol 2018;11. Epub ahead of print Nov 1, 2018. Available from doi:10.1038/mi.2017.74.

Code packages

47. **Kennedy NA**. firsttable R package. Available from https://github.com/NikNakk/firsttable
48. **Kennedy NA**. forestmodel R package. Available from https://cran.r-project.org/package=forestmodel

Oral presentations

49. **Kennedy NA**, Heap G, Hamilton B, Walker GJ, Bewshea C, Bouri S, et al. Clinical effectiveness, safety and immunogenicity of anti-TNF therapy in Crohn's disease: 12-month data from the PANTS study. J Crohn's Colitis 2018;12:S021–2.

50. Sazonovs A, **Kennedy NA**, Bewshea C, Moutsianas L, Walker GJ, De Lange K, et al. Hla-dqa1 contributes to the development of antibodies to anti-TNF therapy in crohn's disease. Gut 2018;67:A52–3.

51. **Kennedy NA**, Warner B, Johnston E, Flanders L, Hendy P, Ding N, Harris R, Fadra AS, Basquill C, Lamb CA, et al. DOP035. Anti-TNF withdrawal in IBD: Relapse and recapture rates and predictive factors from 160 patients in a pan-UK study. J Crohn's Colitis. 2015;9(Suppl 1):S41–2. doi:10.1093/ecco-jcc/jju027.063

52. **Kennedy N**, Warner B, Johnston E, Basquill C, Harris R, Lamb C, Singh A, Fadra A, Cameron F, Basavaraju U, et al. OC-001 Anti-tnf Withdrawal In Ibd: Initial Results From A Pan-UK Study. Gut. 2014 Jun 9;63(Suppl 1):A1–A1. doi:10.1136/gutjnl-2014-307263.1

53. **Kennedy NA**, Adams AT, Hansen R, Ventham NT, O'Leary KR, Drummond H, Noble CL, El-Omar E, Russell RK, Wilson DC, et al. DOP010 Epigenome-wide analysis in childhood-onset Crohn's disease implicates MIR21 in pathogenesis and identifies multiple methylation-based diagnostic biomarkers. J Crohn's Colitis. 2014;8:S19. doi:10.1016/S1873-9946(14)60035-0

54. **Kennedy NA**, Chang J, Guy M, Smith T, Loh JT, Haunschmidt D, Muscat M, Fascì-Spurio F, Drummond HE, Kingstone K, et al. 564 Elevated Faecal Calprotectin Predicts Disease Progression in Crohn's Disease. Gastroenterology. American Gastroenterological Association (AGA); 2013;

Poster presentations (and oral presentations given by others with me as co-author)

55. Heerasing N, Hendy PJ, Moore L, Walker GJ, Bewshea C, Ahmad T, et al. Prevalence and phenotype of inflammatory bowel disease across primary and secondary care: Implications for colorectal cancer surveillance. J Crohn's Colitis 2018;12:S512-3.
56. Walker G, Harrison J, Voskuil M, Heap G, Heerasing N, Hendy P, et al. NUDT15 variants contribute to thiopurineinduced myelosuppression in European populations. J Crohn's Colitis 2018;12:S025-6.
57. Sazonovs A, **Kennedy NA**, Bewshea C, Moutsianas L, Walker GJ, De Lange K, et al. HLA-DQA1 contributes to the development of antibodies to anti-TNF therapy in Crohn's disease. J Crohn's Colitis 2018;12:S009-S010.
58. Green H, Hamilton B, Walker G, Goodhand J, **Kennedy N**, Ahmad T, et al. Gwas of microscopic colitis in the UK biobank confirms association within the hla region. Gut 2018;67:A182-3.
59. Hamilton B, Heerasing N, Hendy PJ, Moore L, Walker GJ, Bewshea C, et al. Prevalence and phenotype of IBD across primary and secondary care: Implications for colorectal cancer surveillance. Gut 2018;67:A67.
60. **Kennedy N**, Heap G, Walker G, Bewshea C, Bouri S, Goodhand J, et al. Clinical effectiveness, safety and immunogenicity of anti-TNF therapy in crohn's disease: 12-month data from pants. Gut 2018;67:A59.
61. Sazonovs A, **Kennedy NA**, Bewshea CM, Moutsianas L, Walker GJ, De Lange K, et al. 590 - HLA-DQA1 Contributes to the Development of Antibodies to Anti-TNF Therapy in Crohn's Disease. Gastroenterology 2018;154:S-124-S-125.
62. Heerasing N, Hendy PJ, Moore L, Walker GJ, Bewshea CM, Ahmad T, et al. Su1875 - Prevalence and Phenotype of Inflammatory Bowel Disease Across Primary and

Secondary Care: Implications for Colorectal Cancer Surveillance. Gastroenterology 2018;154:S-615-S-616.

63. **Kennedy NA**, Heap GA, Hamilton B, Walker GJ, Bewshea CM, Bouri S, et al. 159 - Clinical Effectiveness, Safety and Immunogenicity of Anti-TNF Therapy in Crohn's Disease: 12 Month Data from the Pants Study. Gastroenterology 2018;154:S-44.
64. Walker GJ, Harrison JW, Voskuil MD, Heap GA, Heerasing N, Hendy PJ, et al. 472 - NUDT15 Variants Contribute to Thiopurine-Induced Myelosuppression in European Populations. Gastroenterology 2018;154:S-111.
65. Kalla R, Adams A, Vatn S, Bonfiglio F, Nimmo E, **Kennedy N**, et al. Epigenetic alterations at diagnosis predict susceptibility, prognosis and treatment escalation in inflammatory bowel disease and IBD character. Gastroenterology 2017;152:S565.
66. Kalla R, Adams A, Vatn S, Bergemalm D, Ricanek P, Lindstrom J, et al. Proximity extension assay based proteins show immune cell specificity and can diagnose and predict outcomes in inflammatory bowel diseases: IBD character study. Gastroenterology 2017;152:S606-7.
67. Ho G-T, Aird R, Boyapati RK, Liu B, **Kennedy N**, Shimizu T, et al. MDR1-deficiency unmasks mitochondrial dysfunction as a pathogenic mechanism in IBD. Gastroenterology 2017;152:S962-3.
68. Hendy P, Heerasing N, Walker G, Heap G, Bewshae C, **Kennedy N**, et al. Clinical features of demyelination following anti-TNFalpha therapy. J Crohn's Colitis 2017;11:S360.
69. Adams A, Kalla R, Vatn S, Bonfiglio F, Nimmo E, **Kennedy N**, et al. Epigenetic alterations at diagnosis predict susceptibility, prognosis and treatment escalation in inflammatory bowel disease-IBD Character. J Crohn's Colitis 2017;11:S108.

70. Moutsianas L, De Lange K, Luo Y, Lee J, Jostins L, Lamb C, et al. Whole genome sequencing and imputation in inflammatory bowel disease identifies 26 novel loci and offers therapeutically-relevant mechanistic insights. J Crohn's Colitis 2017;11:S1.

71. Heerasing N, Thompson B, Hendy P, Heap G, Walker G, Bethune R, et al. Exclusive enteral nutrition provides an effective bridge to safer interval elective surgery for Crohn's disease. J Crohn's Colitis 2017;11:S287.

72. Kalla R, Adams A, Vatn S, Bergemalm D, Ricanek P, Lindstrom J, et al. Proximity extension assay based proteins show immune cell specificity and can diagnose and predict outcomes in inflammatory bowel diseases: IBD Character study. J Crohn's Colitis 2017;11:S13.

73. Ho G-T, Aird R, Boyapati R, Liu B, **Kennedy N**, Shimizu T, et al. MDR1-deficiency unmasks mitochondrial dysfunction as a pathogenic mechanism in IBD. J Crohn's Colitis 2017;11:S97.

74. Walker GJ, Harrison JW, Heap GA, Heerasing N, Hendy PJ, Bewshea C, et al. Nudt15 variants contribute to thiopurine induced myelosuppression in European populations. Gut 2017;66:A23-4.

75. Kalla R, Adams AT, Vatn S, Bonfiglio F, Nimmo ER, **Kennedy NA**, et al. Epigenetic alterations at diagnosis predict susceptibility, prognosis and treatment escalation in inflammatory bowel disease-IBD character. Gut 2017;66:A24-5.

76. Walker GJ, Moore L, Heerasing N, Hendy PJ, Bewshea C, Goodhand JR, et al. Clinical validity and utility of faecal calprotectin in primary care. Gut 2017;66:A252.

77. Hendy P, Heerasing N, Walker G, Heap G, Bewshea C, **Kennedy N**, et al. Clinical features of demyelination during anti-TNF therapy: Preliminary outcomes of the PRED4 study. Gut 2017;66:A159.

78. Kalla R, Adams AT, Vatn S, Bergemalm D, Ricanek P, Lindstrom JC, et al. Proximity extension assay based proteins show immune cell specificity and can diagnose and predict outcomes in inflammatory bowel diseases: IBD character study. Gut 2017;66:A202-3.

79. Ventham N, **Kennedy N**, Adams A, Kalla R, Heath SC, O'Leary K, et al. Comprehensive epigenome-wide dna methylation profiling in inflammatory bowel disease. Gastroenterology 2016;150:S156-7.

80. Arnott I, Mowat C, Ennis H, Keerie C, Lewis S, **Kennedy N**, et al. The toppic trial: A randomized, double-blind parallel group trial of mercaptopurine vs placebo to prevent recurrence of crohn's disease following surgical resection in 240 patients. Gastroenterology 2016;150:S182.

81. Kalla R, Ocklind A, Petren C, Nordberg N, **Kennedy N**, Ventham N, et al. Pea immunoassay technology identifies novel serum biomarkers that can diagnose and classify inflammatory bowel diseases: IBD character consortium. Gastroenterology 2016;150:S550.

82. Ventham N, **Kennedy N**, Adams A, Kalla R, Heath S, O'Leary K, et al. Comprehensive epigenome-wide DNA methylation profiling in inflammatory bowel disease. J Crohn's Colitis 2016;10:S4-5.

83. Kalla R, Ocklind A, Petren C, Nordberg N, **Kennedy N**, Ventham N, et al. Proximity extension assay immunoassay technology identifies novel serum biomarkers that can diagnose and classify inflammatory bowel diseases: IBD Character Consortium. J Crohn's Colitis 2016;10:S82.

84. Arnott I, Mowat C, Ennis H, Keerie C, Lewis S, **Kennedy N**, et al. The TOPPIC trial: A randomised, double-blind parallel-group trial of mercaptopurine versus placebo to

prevent recurrence of Crohn's disease following surgical resection in 240 patients. J Crohn's Colitis 2016;10:S21-2.

85. Kalla R, **Kennedy NA**, Ventham NT, Boyapati R, Adams AT, Nimmo E, et al. Serum calprotectin - A novel diagnostic and prognostic marker in inflammatory bowel diseases. Gut 2016;65:A150.
86. **Kennedy NA**, Warner BD, Johnston E, Flanders L, Hendy PA, Ding NS, et al. Anti-TNF withdrawal in IBD: Relapse and recapture rates and predictive factors from 160 patients in a pan-UK study. Gastroenterology 2015;148:S62.
87. **Kennedy NA**, Ennis H, Gaya DR, Mowat C, Arnott ID, Satsangi J. Interobserver agreement in assessment of rutgeerts' score of endoscopic recurrence of ileal crohn's disease. Gastroenterology 2015;148:S431.
88. Kalla R, **Kennedy NA**, Hjelm FN, Modig E, Sundell M, Soderholm J, et al. Proximity extension assay technology identifies novel serum biomarkers for predicting inflammatory bowel disease: IBD character consortium. Gastroenterology 2015;148:S436.
89. Akmacic IT, Ventham NT, Theodoratou E, Vuckovic F, **Kennedy NA**, Kristic J, et al. Inflammatory bowel disease associates with pro-inflammatory potential of the IgG glycome. Gastroenterology 2015;148:S440.
90. **Kennedy NA**, Walker AW, Berry SH, Lamb CA, Lewis S, Mansfield JC, et al. The impact of NOD2 variants on gut microbiota in Crohn's disease and healthy controls. Gastroenterology 2015;148:S718.
91. **Kennedy NA**, Warner B, Johnston E, Flanders L, Hendy P, Ding N, et al. Anti-TNF withdrawal in IBD: Relapse and recapture rates and predictive factors from 160 patients in a pan-UK study. J Crohn's Colitis 2015;9:S41-2.

92. Akmacic IT, Ventham N, Theodoratou E, Vuckovic F, **Kennedy N**, Kristic J, et al. Inflammatory bowel disease associates with pro-inflammatory potential of the IgG glycome. J Crohn's Colitis 2015;9:S107.

93. Kalla R, **Kennedy N**, Hjelm F, Modig E, Sundell M, Andreassen B, et al. Proximity extension assay technology identifies novel serum biomarkers for predicting inflammatory bowel disease: IBD character consortium. J Crohn's Colitis 2015;9:S146-7.

94. **Kennedy NA**, Ennis H, Gaya DR, Mowat C, Arnott IDR, Satsangi J. Interobserver agreement in assessment of Rutgeerts' score of endoscopic recurrence of ileal Crohn's disease: A substudy of the TOPPIC trial. J Crohn's Colitis 2015;9:S231-2.

95. Merrick V, Henderson P, **Kennedy N**, Rogers P, Arnott I, Satsangi J, et al. Natural history of paediatric IBD around transition to adult services: A regional cohort study. J Crohn's Colitis 2015;9:S419-20.

96. **Kennedy NA**, Lindsay JO, Gordon J, Hart A, McCartney S, Irving P, et al. Withdrawal of anti-TNF following sustained remission for inflammatory bowel disease: A systematic review and meta-analysis. Gut 2015;64:A5.

97. Ventham NT, **Kennedy NA**, Adams AT, Kalla R, O'Leary KR, Wilson DC, et al. Epigenome-wide DNA methylation profiling in inflammatory bowel disease. Gut 2015;64:A5-6.

98. Kalla R, **Kennedy NA**, Hjelm F, Modig E, Sundell M, Soderholm J, et al. Proximity extension assay technology identifies novel serum biomarkers for predicting inflammatory bowel disease: IBD character consortium. Gut 2015;64:A8.

99. Pollington C, Cameron FL, **Kennedy NA**, Arnott I, Satsangi J, Wilson D C. Long term outcomes after transition to adult services in paediatric-onset inflammatory bowel disease patients treated with anti-TNF. Gut 2015;64:A97-8.

100. Singh P, Noble CL, Shand AG, Arnott ID, Lees CW, Satsangi J, et al. Initial experience of thiopurine metabolite measurements in the management of IBD in Edinburgh. Gut 2015;64:A242-3.

101. **Kennedy NA**, Ennis H, Gaya DR, Mowat C, Arnott IDR, Satsangi J. Interobserver agreement in assessment of rutgeerts' score of endoscopic recurrence of ileal crohn's disease. Gut 2015;64:A243.

102. Inglis S, **Kennedy N**, Plevris J N. Review of faults and usage of two upper and lower gastrointestinal endoscopes suppliers in NHS Lothian over a 40 month period. Gut 2015;64:A417-8.

103. Kalla R, **Kennedy NA**, Ventham NT, Visconti M, Adams A, Jarvie A, et al. The utility of serum calprotectin as a biomarker in predicting inflammatory bowel disease. Gut 2015;64:A433.

104. Noble CL, Church NI, Shand AG, Lees CW, Brodie H, Greenhill G, et al. PTH-028 Analysis Of Quality Outcomes Following Changing Bowel Preparation For Colonoscopy From Picolax To Moviprep In Nhs Lothian. Gut 2014;63:A220-1.

105. Fasci-Spurio F, **Kennedy N**, Wong L, MacLean P, Satsangi J, Glancy S, et al. PTU-081 Faecal Calprotectin And Ileal Crohn's Disease: Correlation With A Small Bowel Mri Score For Disease Activity. Gut 2014;63:A74-5.

106. Ventham N, **Kennedy N**, Duffy A, Clark D, Crowe A, Knight A, et al. PTU-107 Comparison Of Mortality Following Hospitalisation For Ulcerative Colitis In Scotland Between 1998-2000 And 2007-2009. Gut 2014;63:A86-A86.

107. **Kennedy N**, Walker A, Berry S, Lamb C, Lewis S, Mansfield J, et al. PWE-082 The Impact Of Nod2 Variants On Gut Microbiota In Crohn's Disease And Healthy Controls. Gut 2014;63:A159-60.

108. Ventham N, **Kennedy N**, Duffy A, Clark D, Crowe A, Knight A, et al. PWE-084 Nationwide Linkage Analysis In Scotland - Has Mortality For Hospital Admission For Crohn's Disease Changed In The 21st Century? Gut 2014;63:A160-1.

109. Noble CL, Church NI, Shand AG, Lees CW, Brodie H, Greenhill G, et al. PTH-028 Analysis Of Quality Outcomes Following Changing Bowel Preparation For Colonoscopy From Picolax To Moviprep In Nhs Lothian. Gut 2014;63:A220-1.

110. Fasci-Spurio F, **Kennedy N**, Wong L, MacLean P, Satsangi J, Glancy S, et al. PTU-081 Faecal Calprotectin And Ileal Crohn's Disease: Correlation With A Small Bowel Mri Score For Disease Activity. Gut 2014;63:A74-5.

111. Ventham N, **Kennedy N**, Duffy A, Clark D, Crowe A, Knight A, et al. PTU-107 Comparison Of Mortality Following Hospitalisation For Ulcerative Colitis In Scotland Between 1998-2000 And 2007-2009. Gut 2014;63:A86-A86.

112. **Kennedy N**, Walker A, Berry S, Lamb C, Lewis S, Mansfield J, et al. PWE-082 The Impact Of Nod2 Variants On Gut Microbiota In Crohn's Disease And Healthy Controls. Gut 2014;63:A159-60.

113. Chang J, **Kennedy N**, Fasci Spurio F, Muscat M, Guy M, Satsangi J, et al. Correlation of clinical symptoms to current biomarkers of intestinal inflammation in patients with Crohn's disease. J Crohn's Colitis 2013;7:S116. 1. Ventham N, **Kennedy N**, Duffy A, Clark D, Crowe A, Knight A, et al. PWE-084 Nationwide Linkage Analysis In Scotland - Has Mortality For Hospital Admission For Crohn's Disease Changed In The 21st Century? Gut 2014;63:A160-1.

114. Muscat M, **Kennedy NA**, Chang J, Spurio FF, Satsangi J, Arnott ID, et al. P138 The Prevalence of Anaemia in Inflammatory Bowel Disease in Relation to Disease Activity, As Stratified by Faecal Calprotectin. J Crohn's Colitis 2013;7:S137.

115. **Kennedy N** a., Chang J, Guy MH, Smith T, Loh JT, Haunschmidt D, et al. P250 Elevated Faecal Calprotectin Predicts Disease Progression in Crohn's Disease. J Crohn's Colitis 2013;7:S109-110.

116. **Kennedy NA**, Walker A, UK IBD Microbiota Consortia, UK IBD Genetics Consortia, Berry SH, Duncan SH, et al. P705 Evaluation of the Impact of Different Commercially Available DNA Extraction Kits and Laboratories for Assessing Bacterial Community Structure in Fecal Samples - Implications for IBD Research. J Crohn's Colitis 2013;7:S294.

117. **Kennedy NA**, Chang J, Guy MH, Smith T, Loh JT, Haunschmidt D, et al. Elevated faecal calprotectin predicts disease progression in crohn's disease. Gut 2013;62:A7.

118. **Kennedy NA**, Hildebrand D, Berry SH, Satsangi J, Hold GL. PWE-108 Assessment of the Mucosal Microbiota in Inflammatory Bowel Disease. Gut 2013;62:A174-5.

119. Mattinson A, Hauser B, Gillett H, Gillett P, Goddard C, **Kennedy N**, et al. The FRAX algorithm is of limited utility in predicting osteoporosis in coeliac disease. Gut 2013;62:A263.

120. **Kennedy NA**, Chang J, Guy M, Smith T, Loh JT, Haunschmidt D, et al. 564 Elevated Faecal Calprotectin Predicts Disease Progression in Crohn's Disease. Gastroenterology 2013;144:S-105.

121. Ventham NT, **Kennedy NA**, Nimmo ER, Satsangi J. Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. Gastroenterology 2013;145:293-308.

122. Chang J, **Kennedy NA**, Spurio FF, Muscat M, Guy M, Smith T, et al. Correlation of clinical symptoms to current biomarkers of intestinal inflammation in patients with crohn's disease. Gastroenterology 2013;144:S770.

123. Muscat M, **Kennedy NA**, Chang J, Spurio FF, Satsangi J, Arnott ID, et al. Mo1260 The Prevalence of Anaemia in Inflammatory Bowel Disease in Relation to Disease Activity, As Stratified by Faecal Calprotectin. Gastroenterology 2013;144:S-621.

124. **Kennedy NA**, Reynolds S, Dattani R, Nayee H, Felwick R, Harris R, et al. Su1228 Thiopurine Withdrawal for Sustained Remission in IBD: A UK Multicentre Study. Gastroenterology 2013;144:S-433-S-434.

125. Chang J, **Kennedy NA**, Spurio FF, Muscat M, Guy M, Smith T, et al. Tu1132 Correlation of Clinical Symptoms to Current Biomarkers of Intestinal Inflammation in Patients With Crohn's Disease. Gastroenterology 2013;144:S-770.

126. **Kennedy NA**, Walker A, UK IBD Microbiota Consortia, UK IBD Genetics Consortia, Berry SH, Duncan SH, et al. Tu1716 Evaluation of the Impact of Different Commercially Available DNA Extraction Kits and Laboratories for Assessing Bacterial Community Structure in Fecal Samples - Implications for IBD Research. Gastroenterology 2013;144:S-829.

1 Introduction

1.1 Crohn's disease

Crohn's disease is a chronic condition characterised by inflammation of the gastrointestinal tract.[1] It is one of the two main subtypes of inflammatory bowel disease (IBD). One of the earliest descriptions of Crohn's disease was in 1913 by Thomas Kennedy Dalziel, a surgeon working in Glasgow who had studied medicine at the University of Edinburgh.[2] He described three cases of a chronic intestinal inflammation that he had originally presumed to be tuberculous, but which was negative on microbiological examination. He described the affected bowel as having the 'consistence and smoothness an eel in a state of rigor mortis'. He also noted that while he '[regretted] that the etiology of the condition remain[ed] in obscurity, [he trusted] that ere long further consideration will clear up the difficulty'. Crohn, Ginzburg and Oppenheimer later published their description of this disease as a distinct pathological entity in 1932[3] and the disease was subsequently named after Burrill Crohn.

For rather longer than Dalziel had perhaps anticipated, the cause of Crohn's disease remained poorly understood. However, the last two decades have seen important advances in our understanding of the pathogenesis of Crohn's disease, and it is now commonly thought to result from an aberrant immune response to gastrointestinal microbiota resulting from complex interactions between dietary, environmental and genetic factors. The importance of environmental influences on disease risk has been highlighted by the change in incidence and prevalence globally. The incidence in the Western world has steadily risen over the past five decades,[4,5] and we have now also seen a rapid rise in

incidence in other countries such as China associated with a Westernisation of diet and lifestyle.[5,6]

1.2 Aetiology of Crohn's disease

The genetic contribution to the aetiology of Crohn's disease were first demonstrated by twin and other family studies in the 1980s and 1990s.[7] Since then, our knowledge and understanding of the variants associated with Crohn's disease has grown as the technology required to analyse the genome has developed, from genetic linkage through genome wide association studies to genome sequencing.[8–12] *NOD2* was the first genetic association described with Crohn's disease and remains one of the strongest genetic predictors of developing disease.[13] It is an intracellular innate immune protein that includes a pattern-recognition receptor to identify bacterial peptides and initiate immune responses.[14] Mice who are deficient in *NOD2* do not spontaneously develop colitis, but are more susceptible to intestinal infection with *Listeria monocytogenes*.[15]

There are now known to be more than 200 loci associated with inflammatory bowel disease (IBD), most shared between Crohn's disease and ulcerative colitis, and most of small effect size.[10,12,16] These studies have highlighted the importance of innate immunity and mechanisms such as autophagy and T-helper-17 cells in pathogenesis. However, the effect size of most individual genetic variants is relatively small. Genetic risk scores may help integrate information from across multiple loci to improve predictive power, but even with a combined odds ratio of 3, they are not sufficient to identify future IBD risk in an individual with enough certainty to intervene.[17] Genetic associations have also been identified for Crohn's disease phenotype, particularly disease location, in a large study conducted by the international IBD genetics consortium.[18] Interestingly, genetic associations with disease

behaviour independent of location were not identified, suggesting that differences in disease behaviour may reflect environmental influence.

More recently, the importance of the gastrointestinal microbiota have been recognised in Crohn's disease.[19,20] Scientists have expended much effort searching for a single causative pathogen. Some candidates such as adherent-invasive *Escherichia coli* have shown clear associations with certain disease phenotypes in subsets of patients,[21] but in general it is now recognised that the role of bacteria and other micro-organisms in Crohn's disease pathogenesis is more complex. [19,20] Early studies of the microbiota in Crohn's disease used culture-dependent techniques and were therefore limited by the difficulties in growing predominantly anaerobic intestinal bacteria *ex vivo*. The advent of nucleic-acid-based methods for assaying microbiota has subsequently greatly increased our ability to interrogate bacteria, viruses and fungi at both taxonomic and genomic levels. Sequencing of variable regions of marker genes, typically the 16S rRNA gene for bacteria, permits relatively affordable taxonomic surveys of faecal and mucosal samples. Where feasible, a deeper understanding of the functional aspects of the gut microbiota can be obtained using shotgun metagenomic sequencing.[22,23]

Methodological aspects of studies of the gut microbiota are of key importance. It is well-recognised that the results of such studies are influenced by initial sample handling and storage, choice of extraction kit and method and for marker gene studies, the region of the gene chosen for study.[24–26] Consistency within an individual study is essential to avoid introducing bias or reducing the power to find associations, but it is also important to validate the methods used, particularly when planning larger scale multisite studies.

The third key player in pathogenesis of Crohn's is the environment. The rise in incidence, particularly now in emerging economies,[27] cannot be explained by genetics. It is probable that shifts in gut microbiota have played a role, but the driving influence is likely to be changes in diet and other environmental factors.[28,29] The best evidence for an environmental risk factor in Crohn's is for tobacco smoking.[30–34] Definitive evidence for which dietary components modify risk has been harder to determine, in part reflecting greater complexity. However, there is emerging evidence for dietary emulsifiers including compelling data from mouse models.[35]

Epigenetics has emerged as a potential mechanism that may explain how the environment and microbiota may interact with host genetics and lead to changes in gene expression.[36] Epigenetic modifications are heritable modifications that alter gene expression without changes in the underlying DNA sequence. DNA methylation remains the most well-studied epigenetic modification, and there has been growing interest in its role in pathogenesis of complex diseases including inflammatory bowel disease[37–40] as well as other immune-mediated diseases[41,42]. Other epigenetic factors that may be relevant to the pathogenesis of Crohn's disease include micro-RNAs and histone modifications.[36,43]

1.3 Diagnosis of Crohn's disease

Crohn's disease has an estimated population prevalence in Western Europe and the United States of 100-300 per 100,000 population.[5] The most recent estimate of prevalence from Edinburgh is 284/100,000 (1 in 353 people).[44] It can affect the entire gastrointestinal tract, from mouth to anus. The most common site is the terminal ileum; in a large international genotype-phenotype analysis 75% of 16,902 Crohn's patients had ileal or ileocolonic involvement.[18] Clinical presentations vary widely, but the most common symptoms are abdominal pain, chronic diarrhoea and weight loss.[45]

Delay in diagnosis has been associated with poorer outcome,[46] and so it is important to ensure patients with Crohn's disease have an early referral to secondary care gastrointestinal services and investigation. However, gastrointestinal symptoms are a common presentation in primary care, and irritable bowel syndrome has an estimated population prevalence of 11%.[47] Distinguishing functional bowel diseases such as irritable bowel syndrome from organic intestinal diseases such as Crohn's disease can be challenging. Some patients with Crohn's disease will have unambiguous evidence on routine blood tests of an inflammatory process, but in others the C-reactive protein (CRP), albumin and haemoglobin may be within normal limits. Definitive diagnosis of Crohn's disease can only be made using endoscopy and/or cross-sectional imaging.[48] However, endoscopic techniques are invasive, cross-sectional imaging may involve ionising radiation and both are expensive. There is therefore a need for affordable non-invasive biomarkers to permit stratification of patients with symptoms suggestive of Crohn's disease into those at higher and lower risk of having the disease.

Faecal calprotectin was first described as a potential biomarker of gastrointestinal disease in the early 1990s.[49] It is a 36.5 kDa calcium-binding protein that is the most abundant cytosolic protein in neutrophils, but is also released by other immune cells such as macrophages. Faecal calprotectin has been shown to correlate with endoscopically-determined intestinal inflammation[50–54], and is increasingly used as a non-invasive test to guide diagnosis of inflammatory bowel disease[55–58] and also for monitoring of established disease.[59,60]Crohn's disease phenotype

Patients with Crohn's disease display a broad range of phenotypes, some of which is now known to be influenced by genetic variation.[18] The most widely-used classification for Crohn's disease phenotypes at present is the Montreal classification[61] (Table 1-1) Accurate

phenotyping requires ileocolonoscopy and small bowel imaging, now most commonly magnetic resonance enterography. In adults, upper gastrointestinal (GI) endoscopy is usually reserved for patients who have symptoms consistent with upper GI involvement. Disease location is relatively stable over time, whereas most patients with Crohn's disease progress from inflammatory to structuring and/or penetrating disease.[18,62]

Table 1-1 Montreal classification of Crohn's disease

Age at diagnosis	
A1	≤16 years
A2	17-40 years
A3	>40 years
Disease location	
L1	Distal ileum ± caecal involvement
L2	Isolated colonic disease
L3	Ileocolonic (distal ileum + colon beyond caecum)
L4	Upper GI (proximal ileum upwards). L4 can also be used as a modifier for L1/2/3
Disease behaviour	
B1	Inflammatory disease: non-stricturing, non-penetrating
B2	Stricturing disease: constant luminal narrowing with pre-stenotic dilatation or obstructive signs/symptoms
B3	Penetrating: bowel perforation, intra-abdominal fistulas, inflammatory masses or abscesses not related to post-op complications. Excludes isolated perianal or rectovaginal fisulae
p modifier	Perianal abscesses, ulcers or fistulas (but not skin tags or fissures)

1.4 Treatment of Crohn's disease

The earliest reports of treatment for Crohn's disease were surgical[2], but there are now a wide range of therapies available, and patients are managed by a multidisciplinary team (MDT) involving physicians, surgeons, nurses, dieticians and pharmacists. The MDT works with the patient and aim to choose the optimum treatment for that patient at that stage of their disease, although evidence to guide personalisation of therapy is still limited.

1.4.1 Dietary therapies

In light of the epidemiological associations between shifting patterns of diet and incidence of Crohn's disease, it is attractive to consider whether manipulation of diet can be used to treat the disease. The best data are for use of exclusive enteral nutrition (EEN) in children, where meta-analysis has demonstrated remission rates of 73%.[63] In this therapy, patients receive all of their nutrition from liquid food replacements, typically polymeric formulations. The data in adults are more mixed, although there is evidence to indicate that when adult Crohn's disease patients are able to tolerate EEN, it is effective.[63–67] It can be considered as an alternative to corticosteroids in patients with mild-to-moderate Crohn's disease, particularly in patients who wish to avoid the potential adverse effects of corticosteroids. EEN may have a role in the optimisation of patients prior to surgery for Crohn's disease, and in fact in some patients may obviate the need for surgery altogether.[68]

More recently, groups in Glasgow and in Israel have published data on using partial enteral nutrition alongside a specially modified diet to induce remission for Crohn's disease.[69–71] Early results are promising, suggesting that it is at least as effective as exclusive enteral nutrition but is better tolerated.

1.4.2 Corticosteroids

One of the oldest treatment classes used for Crohn's disease, corticosteroids continue to have a place in the induction of remission. In patients with mild-to-moderate ileocaecal disease, there is good evidence from meta-analysis for the use of the budesonide.[72] Given orally, budesonide is released in the small bowel and has its effect through local action in the distal small bowel and proximal colon. 90% of the drug is broken down by first pass metabolism in the liver, so the incidence of side effects is lower than for systemically-acting steroids such as prednisolone.

For patients with more severe disease or with colonic involvement beyond the ascending colon, the most widely-used steroid used for induction of remission is prednisolone, with good evidence for effect.[73] However, adverse effects are common, and guidelines recommend minimising corticosteroid use where possible.[74] Nonetheless, corticosteroids remain a commonly prescribed therapy, and use of steroids in excess of guidelines was seen in almost 15% of patients in a UK-based study of 2385 patients.[75]

1.4.3 Immunomodulators

Azathioprine and its metabolite mercaptopurine are thiopurines that were originally developed in the 1950's for the treatment of leukaemia. Their use in inflammatory bowel disease were first described for ulcerative colitis in the 1960's[76], and the earliest trials in Crohn's disease were published in the 1970's.[77] Azathioprine is metabolised to mercaptopurine. Mercaptopurine itself is then processed via three separate metabolic pathways leading to thiouric acid, the methylated mercaptopurine nucleotides (MMPN) and thioguanine nucleotides (TGN).[78] There is significant interindividual variability in this metabolism. One of the key enzymes in the MMPN pathway is thiopurine methyltransferase (TPMT), and TPMT activity testing was the first pharmacogenetic test to be introduced into routine IBD practice.

The active metabolites, TGN, are believed to work through inhibition of Rac1 which induces apoptosis of T-cells and immunosuppression.[78] Perhaps as a result of this, the onset of action of thiopurines is slow, typically 8-12 weeks. When last reviewed by Cochrane, thiopurines were found to be effective for maintenance of remission in Crohn's,[79] but not induction of remission.[80]

Methotrexate inhibits folate metabolism, and it is thought that the resultant inhibition of DNA, RNA and protein synthesis lead to its immunomodulatory effects.[81] Like thiopurines, methotrexate does not have a role in induction of remission, but has been shown in meta-analysis of controlled trials to have a benefit in maintaining remission.[82]

1.4.4 Anti-tumour-necrosis-factor-alpha (anti-TNF) therapy

Infliximab is a monoclonal chimeric antibody that binds to tumour necrosis factor alpha (TNF). It was first successfully used in a small trial of rheumatoid arthritis patients in London in 1992.[83] Although there was use of single doses or short courses of infliximab for Crohn's disease in the late 1990's, the landmark trial in Crohn's disease was the placebo-controlled ACCENT-I trial of maintenance infliximab therapy published in 2002.[84] Adalimumab, a fully human antibody to TNF came slightly later, with the CLASSIC-I study of induction therapy published in2006,[85] and the CHARM study of maintenance therapy the following year.[86] Both drugs are now well-established in the induction and maintenance of remission for Crohn's disease, and NICE recommended their use in 2010.[87]

There is good evidence to support the use of combination therapy for Crohn's disease when using infliximab. The SONIC study demonstrated a significant difference in remission between patients treated with infliximab alone and those treated with infliximab plus azathioprine.[88] The benefits of this approach are less clear with adalimumab, and a recent meta-analysis concluded there was no benefit of using immunomodulators in combination with adalimumab over adalimumab monotherapy.[89]

Traditionally, treatment of Crohn's disease has been using a step-up approach, where more established, cheaper therapies are tried first. The NICE guidance in the UK is that anti-TNF therapies are used in those patients who have failed or are intolerant to conventional

therapies (immunomodulators and steroids).[87] This has been challenged over the past decade. The step-up/top-down study was first presented over ten years ago,[90] showed that early combination therapy ('top-down') led to a higher rate of clinical remission and reduction in corticosteroid use compared with a 'step-up' approach. More recently, long-term data were published on the trial participants.[91] Interestingly, after a median follow-up of 8 years there was no longer a difference in the rates of clinical remission, but there were lower relapse rates and a reduced use of anti-TNF therapy and corticosteroids.

1.4.4.1 Immunogenicity

As exogenous proteins, biologic therapies such as infliximab and adalimumab can lead to the generation by the patient of anti-drug antibodies. This phenomenon is called immunogenicity, and its importance has been increasingly recognised when considering treatment with these drugs.[92] Anti-drug antibodies may lead to increased clearance of the drug or to neutralisation of its effectiveness. As such, they are an important cause of loss of response to anti-TNF therapy.

1.4.4.2 Biosimilars

As indicated in 1.4.4, both infliximab and adalimumab have been in use for over a decade. Infliximab's patent expired in Europe in 2015, while adalimumab's expired in 2018. This has permitted the licensing of 'biosimilar' medicines. Biosimilars are developed to match the reference product as closely as possible in both structure and function.[93] During development, structural aspects that are controlled include the amino-acid sequence, higher order structure, post-translational modifications. Their function is assessed *in vivo*, including receptor binding and triggering of antibody-dependent and complement-dependent cytotoxicity. Presuming they have sufficiently similar structure and function to the reference molecule, they are then studied in comparative clinical studies. These are

typically done in two representative indications, with extrapolation of equivalence made to the other indications. For example, for infliximab, the licensing studies performed were in rheumatoid arthritis and ankylosing spondylitis.[94,95] However, CT-P13 (biosimilar infliximab) received a product licence that matched the reference product, Remicade, and so included ulcerative colitis and Crohn's disease. Post-marketing studies are therefore helpful in assessing whether the equivalence or non-inferiority observed in the pre-marketing studies also applies to the broader range of indications.

1.4.5 Newer medical therapies

Following the successful introduction of anti-TNF for Crohn's disease, there was a relatively long interval before further biologic therapies were licensed in Europe for Crohn's. Integrins and their counterpart, cellular adhesion molecules, represent an attractive drug target to limit trafficking of leukocytes into tissues. Natalizumab, an anti-α4 integrin proved effective in clinical trials, but was found to have a 2 in 1000 chance of progressive multifocal leukoencephalopathy driven by JC polyomavirus reactivation.[96,97] It was therefore refused licensing by the European Medicine Agency's (EMA) Committee for Medicinal Products for Human Use in 2007. Other integrin inhibitors in development, including the gut-specific anti-α4-β7 integrin vedolizumab, underwent further extensive safety testing to demonstrate they were not associated with the same risk,[98] and the landmark phase 3 trial in Crohn's disease was not published until 2013.[99] Vedolizumab was licensed for Crohn's disease in Europe in 2014, and the following year NICE approved its use in patients who had failed anti-TNF therapy or in whom anti-TNFs were contraindicated.[100] Its use in real-world UK settings has now been described by multiple UK-based groups.[101–104] The first head-to-head randomised controlled trial in inflammatory bowel disease has now been published which showed superiority of vedolizumab and adalimumab for treatment of ulcerative

colitis,[105] but no head-to-head studies of this type have yet been published for Crohn's disease.

Interleukins 12 and 23 have both been implicated in the pathogenesis of Crohn's disease and other immune-mediated diseases.[106] IL-12 is involved in the development of T_H1 immune responses, while IL-23 is involved in stabilisation of T_H17 cells. Both IL-12 and 23 share a common p40 subunit. Ustekinumab, a fully human anti-p40 antibody, was first licensed for psoriasis in 2009. Phase 3 trials in Crohn's disease were published in 2016.[107] The EMA approved its use later that year, and NICE approved it in 2017.[108]

There are many other drugs in development for Crohn's disease, including other anti-integrins[97] those targeting the Janus Kinase (JAK) family of enzymes and sphingosine 1 receptor agonists.[109] Some, such as mongersen,[110], an anti-SMAD7 oligonucleotide, and vercinon,[111] a CC chemokine receptor antagonist, have shown promise in phase 2 trials but have not gone on to prove effective in phase 3. However, physicians and patients will be presented with an increasing number of therapeutic options over the next few years, and it will be critical to develop strategies for choosing between them.

1.4.6 Surgery

Crohn's disease has long been a condition that has been managed by both gastroenterologists and surgeons. A recent population-based study demonstrated that by twenty years following diagnosis, almost 60% of patients with Crohn's disease will have had major surgery.[112] Recent UK data suggest a reduction in the rate of surgery since the turn of the 21st century[113], but elective and emergency surgical admissions remain common.

In line with other aspects of general surgery, much progress has been made towards minimally invasive Crohn's surgery, and many operations can be done laparoscopically.[114]

The Lir!c study recently demonstrated that, for limited ileocaecal Crohn's disease, a limited laparoscopic resection is a reasonable alternative to anti-TNF therapy in terms of quality of life.[115] Good multidisciplinary teamworking is central to modern care of Crohn's disease care, so that the full range of medical and surgical options can be considered.

1.5 Predicting and modifying disease course of Crohn's disease

There is a wide spectrum of Crohn's disease severity. Some patients will have a relatively quiescent disease course and achieve control of symptoms and bowel inflammation with a single course of corticosteroids, while other patients have more aggressive disease leading to progressive bowel damage and disability.[116–118] Overall, a majority of patients will progress from inflammatory to stricturing and/or penetrating disease over the course of the first decade of their disease.[62] Although recent studies have identified several clinical and laboratory variables that are associated with disease outcome,[18,119] our ability to accurately predict disease course in an individual patient remains poor.

Historically, management of Crohn's disease is often reactive; escalation of treatment is a response to worsening or persistence of symptoms. There has recently been a shift in emphasis towards aiming for mucosal healing, in recognition of the poorer outcomes experienced by patients with persistent endoscopic inflammation and the progression of bowel damage.[116,120] However, there remains an unmet need for non-invasive biomarkers that identify those patients at greater risk of disease progression so that treatment can be tailored to the individual.

Treatment options for Crohn's disease have greatly expanded over the past twenty years as detailed in section 1.4 above. These drugs have proved to be effective and relatively safe but remain expensive. Current NICE guidelines recommend annual review of patients on

these medications with consideration of withdrawal for patients in stable, clinical remission.[87,100,108] However, in clinical practice, both physicians and patients are often reluctant to cease medications that are perceived to be keeping a patient well. Patients and physicians need accurate data on outcomes of treatment withdrawal to inform such decisions, and ideally to be able to identify clinical features and biomarkers that help predict in which patients drug withdrawal is more likely to be successful.

A majority of patients with Crohn's disease will need one or more resectional operations during their disease course.[112] Although biological therapies have made a marked difference to the lives of patients with Crohn's, they have not obviated the need for surgery. Where possible, the aim of surgery is often to remove all of the segments of inflamed bowel, resulting in surgically-induced remission. However, disease recurrence following surgery is common, with a median time to endoscopic recurrence of six months, clinical recurrence 3-5 years and further surgery 10-20 years.[121] Early identification of patients at risk of recurrence is therefore important, and there is now evidence supporting escalation of therapy driven by detection of endoscopic disease activity.[122] Physicians also then need evidence-based therapies they can use to reduce this risk.

1.6 Summary

Crohn's disease is a chronic immune-mediated condition which can cause marked morbidity and progressive bowel damage. Research is required to improve early diagnosis, and to stratify patients' risk at key points in their disease course including when considering disease withdrawal or in the post-operative setting. Identification of non-invasive biomarkers for this purpose will permit the development of better personalised care.

2 Hypothesis and aims

I hypothesise that biomarkers exist that can be used to improve the diagnosis and management of Crohn's disease. These may include proteins, genetic and microbial biomarkers.

I aim to investigate the role of various biomarkers and to explore how they can be used alongside clinical parameters in the diagnosis and management of Crohn's disease.

3 Methods

3.1 Study designs

When designing studies to assess the impact of particular variables of interest on an outcome, there are a number of different possible approaches. The gold standard for testing hypotheses in medical research are randomised controlled trials (RCTs);[123] However, RCTs are often impractical for assessing the impact of certain exposures on outcomes, either because it is not possible to control the exposure, or it would be unethical or infeasible to do so. Cohort studies offer an alternative approach. A cohort of study participants is defined based on prespecified inclusion and exclusion criteria and followed over time to see whether the outcome in question occurs. Cohort studies permit the exploration of multiple, potentially interacting baseline variables, but it is never possible to completely control for the possibility of residual confounding.

One of the most important considerations when designing a cohort is whether to take a retrospective or prospective approach.[123,124] Both have their potential advantages and disadvantages. Retrospective studies are efficient to perform, since the data required is often already available in electronic patient records and research databases. However, the data concerned has not been collected for the specific purposes of the study. This restricts the choice of variables that can be measured and increases the possibility of missingness of key data. Depending on the method for selecting patients, retrospective cohort studies are also at risk of selection bias. Physicians asked to recall patients exposed to an intervention may be predisposed to remember those who did particularly well or badly. This can be mitigated to some degree by using electronic searches of electronic records to ensure that all patients meeting a set of criteria are included in the study, or where this is not possible that the sample of patients used is chosen at random.

Prospective cohort studies can be costly to run and may take several years to accumulate adequate data, but there is greater control of the variables collected and the quality and uniformity of the data. Since the outcome is unknown at the point of study entry, there is less of an issue of selection bias. Nonetheless, there may still be referral bias with respect to how patients are chosen for the study, and bias from loss-to-follow-up may affect both study designs.

Both approaches have their merits, and in this thesis I include several large retrospective cohort studies which span longer time periods than would have been feasible prospectively, as well as a large multicentre prospective study that benefitted from the greater uniformity of follow-up and more detailed data collection.

3.2 Statistical methods

Through all of the cohort studies presented in this thesis, there is a requirement to describe the distribution of the measured variables and to make comparisons against one of the outcomes of interest. The independent variables are either categorical or continuous. For the continuous variables, they may follow a normal distribution (or a distribution that can be transformed to normal) or some other distribution. Many potential clinical parameters and biomarkers measured in studies such as these do not follow a normal distribution. Some, such as C-reactive protein and faecal calprotectin are closer to a log-normal distribution. However, in general for continuous variables throughout the papers represented in this thesis I have reported medians and interquartile ranges, since these make fewer assumptions of the underlying distribution of the data. For categorical data, I have reported counts and percentages.

For univariable comparative statistics, I have generally opted to use the Mann Whitney U test for comparison of a continuous variable against a binary outcome. I have used Fisher's exact test for comparison of two categorical variables. With respect to multivariable models, I have used logistic regression when using a binary outcome, and linear regression for continuous outcomes. In both cases, I have transformed log-transformed continuous data where it more closely approximates a log-normal distribution.

For some analyses, it is more appropriate to look at the time to an event rather than treating it as a binary outcome. This type of time-to-event or survival approach has the advantage of dealing with loss-to-follow-up, so long as the censoring is non-informative. For estimation of the event rate over time, I have used the Kaplan-Meier method. For univariable analyses, I have used either the log-rank test or Cox proportional hazards, and for multivariable analyses I have used Cox proportional hazards.

When understanding the trade-off between sensitivity and specificity of potential diagnostic biomarkers, receiver operating characteristic (ROC) analysis is helpful. This is also helpful when making comparisons between the performance of such biomarkers. The area under the receiver operating characteristic curve is helpful as a measure here. Delong' s method can be used for comparison between two ROC curves where the direction of the curve is the same.[125] An alternative method that can be used where the direction of the curves is different is bootstrapping.[126]

For almost all of the statistical analyses presented in this thesis, I used the open source statistical computing environment R (R Foundation for Statistical Computing, Vienna, Austria).

4 The Impact of Different DNA Extraction Kits and Laboratories upon the Assessment of Human Gut Microbiota Composition by 16S rRNA Gene Sequencing

4.1 Introduction to paper

Interactions between the host and the intestinal microbiota plays a key role in the pathogenesis of Crohn's disease.[127] As detailed in section 1.2, our understanding of differences in the bacteria that populate the gut in patients with Crohn's disease has rapidly evolved with the advent of molecular techniques that permit detailed interrogation of the bacterial contents of faecal and intestinal samples. Methodological considerations such as the choice of DNA extraction kit are important groundwork for such studies, since it is possible otherwise to over- or under-estimate the proportions of individual genera or even whole phyla. In this paper, I worked with colleagues to assess the impact of two different widely-used extraction kits, the Mobio PowerSoil and MP Biomedicals FastDNA SPIN kit for soil. This work was done in preparation for the next chapter in which we used the second of these kits.

4.2 Contributions

I designed the experiments, alongside Alan Walker, Petra Louis, Sylvia Duncan, Harry Flint, Julian Parkhill, Jack Satsangi, Charlie Lees and Georgina Hold. Initial sample collection and preparation was performed by Susan Berry, Sylvia Duncan, Freda Farquarson and John Thomson. The 16S rRNA pyrosequencing was performed and initially processed by Alan Walker. I performed the quantitative PCR (qPCR) experiments under the supervision of Georgina Hold and Susan Berry. I analysed both the pyrosequencing and qPCR data under

supervision of Alan Walker, Petra Louis and Georgina Hold. Georgina Hold and I wrote the paper.

Published as: **Kennedy NA**, Walker AW, Berry SH, Duncan SH, Farquarson FM, Louis P, et al. The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. PLoS One 2014;9:e88982. Available from https://dx.doi.org/10.1371/journal.pone.0088982.

OPEN ACCESS Freely available online

PLOS ONE

The Impact of Different DNA Extraction Kits and Laboratories upon the Assessment of Human Gut Microbiota Composition by 16S rRNA Gene Sequencing

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Abstract

Introduction: Determining bacterial community structure in fecal samples through DNA sequencing is an important facet of intestinal health research. The impact of different commercially available DNA extraction kits upon bacterial community structures has received relatively little attention. The aim of this study was to analyze bacterial communities in volunteer and inflammatory bowel disease (IBD) patient fecal samples extracted using widely used DNA extraction kits in established gastrointestinal research laboratories.

Methods: Fecal samples from two healthy volunteers (H3 and H4) and two relapsing IBD patients (I1 and I2) were investigated. DNA extraction was undertaken using MoBio Powersoil and MP Biomedicals FastDNA SPIN Kit for Soil DNA extraction kits. PCR amplification for pyrosequencing of bacterial 16S rRNA genes was performed in both laboratories on all samples. Hierarchical clustering of sequencing data was done using the Yue and Clayton similarity coefficient.

Results: DNA extracted using the FastDNA kit and the MoBio kit gave median DNA concentrations of 475 (interquartile range 228-561) and 22 (IQR 9-36) ng/μL respectively ($p<0.0001$). Hierarchical clustering of sequence data by Yue and Clayton coefficient revealed four clusters. Samples from individuals H3 and I2 clustered by patient; however, samples from patient I1 extracted with the MoBio kit clustered with samples from patient H4 rather than the other I1 samples. Linear modelling on relative abundance of common bacterial families revealed significant differences between kits; samples extracted with MoBio Powersoil showed significantly increased *Bacteroidaceae*, *Ruminococcaceae* and *Porphyromonadaceae*, and lower *Enterobacteriaceae, Lachnospiraceae*, *Clostridiaceae*, and *Erysipelotrichaceae* ($p<0.05$).

Conclusion: This study demonstrates significant differences in DNA yield and bacterial DNA composition when comparing DNA extracted from the same fecal sample with different extraction kits. This highlights the importance of ensuring that samples in a study are prepared with the same method, and the need for caution when cross-comparing studies that use different methods.

Citation: Kennedy NA, Walker AW, Berry SH, Duncan SH, Farquarson FM, et al. (2014) The Impact of Different DNA Extraction Kits and Laboratories upon the Assessment of Human Gut Microbiota Composition by 16S rRNA Gene Sequencing. PLoS ONE 9(2): e88982. doi:10.1371/journal.pone.0088982

Editor: Yolanda Sanz, Instutite of Agrochemistry and Food Technology, Spain

Received September 13, 2013; **Accepted** January 13, 2014; **Published** February 24, 2014

Funding: This project was funded by a grant from CORE – The Digestive Disorders Foundation. Funding for AWW, JP, and 454 pyrosequencing was provided by the Wellcome Trust (grant WT076964). NAK is funded by a Research Training Fellowship from the Wellcome Trust (grant number WT097943MA). SHD, FMF, PL, and HJF receive support from the Scottish Government Rural and Environment Science and Analytical Services. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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¶ Membership of the UK IBD Genetics Consortium is provided in the Acknowledgments.

Introduction

The last decade has seen a marked rise in interest in the bacterial communities that coexist within humans, facilitated by the availability of modern molecular techniques. The Human Microbiome Project[1] and MetaHIT[2] have made considerable progress in furthering our understanding of microbial diversity and community structure in different body areas of healthy individuals. The gastrointestinal tract is the most heavily colonized organ in the body, with 70% of bacteria found in humans residing in the colon[3–5]. Differences in the diversity and community structure of the gut microbiota have been associated with diseases of the gastrointestinal tract such as inflammatory bowel disease

(IBD)[6,7] and irritable bowel syndrome[8], as well as metabolic disorders like type 2 diabetes mellitus and obesity[9].

Determining the bacterial community structure in fecal samples through amplification and sequence analysis of extracted DNA has revolutionized gastrointestinal microbiology research over recent years. These culture-independent techniques for assessing diversity have largely replaced traditional culture based approaches as they are considered to be less biased in terms of defining true diversity and considerably less labor-intensive[10,11]. Due to the recent rapid increase in DNA-based phylogenetics of bacterial communities many different DNA extraction procedures are used, each with its own potential biases. All methods rely on chemical or mechanical disruption, lysis using detergents, or a combination of these approaches.

Previous studies have evaluated differences between DNA extraction methods from fecal samples, exploring detection with conventional PCR[12,13], quantitative PCR[14,15], bands on denaturing gradient gel electrophoresis (DGGE)[15–20] and phylogenetic microarray[21]. Significant differences in relative abundance have been demonstrated when DNA was extracted using different methods from mock communities of bacteria and assessed by 16S rRNA sequencing[22,23]. Wu *et al.* described the effect of different fecal extraction methods on 16S rRNA pyrosequencing, comparing QIAamp DNA Stool Minikit, MoBio PowerSoil DNA Isolation Kit and Stratec PSP Spin Stool DNA Kit[24].

The aim of this study was to analyze bacterial communities in healthy volunteer and IBD patient fecal samples extracted using the MoBio and FastDNA DNA extraction kits in two established gastrointestinal research laboratories. The MoBio Power Soil DNA extraction kit and the MP Biomedicals Fast DNA Spin Kit for Soil DNA extraction kit are two commonly used extraction procedures for fecal microbial diversity studies[25–27]. Both methods use a combination of mechanical disruption and chemical lysis.

Methods

Fecal sample collection and initial processing

Fecal samples were taken from two patients with IBD (I1 and I2) and from two healthy controls (H3 and H4) using the Fisher Fecal Commode Collection Kit. Fecal samples were kept at 4°C and processed within 4 hours of collection. This short period of storage is not expected to influence molecular estimation of microbial community composition[25]. Each sample was thoroughly mixed and several aliquots of 500 mg were dispensed. Aliquots were distributed between two established microbial research laboratories (Institute of Medical Sciences (IMS) and The Rowett Institute of Nutrition and Health (RINH), both Aberdeen University) and then subject to further processing as detailed in Figure 1 and described below.

Ethics Statement

Ethical approval was granted by North of Scotland Research Ethics Service (03/0137 and 12/NS/0061) on behalf of all participating centers and written informed consent was obtained from all subjects.

MoBio PowerSoil DNA extraction procedure

One 500 mg fecal aliquot was used for MoBio PowerSoil DNA isolation kit extraction. 5 ml of MoBio lysis buffer was immediately added to the fresh fecal sample, which was then vortex mixed for 30–40 seconds. Fecal suspensions were then centrifuged (1,500 g×5 minutes) and 1 ml of the supernatant placed into the MoBio Garnet bead tubes containing 750 ul of MoBio buffer. These tubes were then heated at 65°C for 10 minutes, then at 95°C for 10 minutes. Samples were then stored at −80°C prior to processing in both laboratories following the manufacturer's instructions. DNA was eluted in 100 µL MoBio elution buffer.

FastDNA SPIN Kit for Soil procedure

For each fecal sample 2×500 mg aliquots were placed in FastDNA SPIN Kit lysing matrix E tubes and 978 µl of sodium phosphate buffer and 122 µl MT buffer were added to each tube and vortex mixed. One aliquot was then stored at −80°C and was defined as FastDNA method 1. The second aliquot was subjected to additional processing by heating at 65°C for 10 minutes, then at 95°C for 10 minutes followed by storage at −80°C. This was defined as FastDNA method 2. Both aliquots were then processed following manufacturer's (Qbiogene, MP Biomedicals, Illkirch, France) instructions. DNA was eluted in 100 µL FastDNA elution buffer.

PCR amplification

Fecal DNA was quantified by Nanodrop mass spectrophotometry before dilution to 25 ng/µl. Initial PCR amplification was undertaken at each laboratory with Invitrogen AccuPrime Taq DNA Polymerase High Fidelity utilising a per-reaction mix of 2 µl of DNA template, 2 µl of Buffer II, 0.2 µl (2 µM) Fusion Primer A, 0.2 µl (2 µM) Fusion Primer B, 0.08 µl (1 U) Accuprime Taq and 15.52 µl sterile, deionized water to a final volume of 20 µl. Quadruplicate PCR reactions were set up per DNA sample. The 16S rRNA gene primers, spanning the V3-5 region of the 16S rRNA gene, were configured as follows: 338F, 5′-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*ACTCCTACGGGAGGCAGCAG-3′, where the bases in italics are 454 Lib-L kit adaptor "B", and 926R, 5′- CCATCTCATCCCTGCGTGTCTCCGACTCAG-Marker-CCGTCAATTCMTTTRAGT-3′, where the underlined bases are 454 adaptor "A" and the marker sequence was a unique 12-mer string of error-correcting Golay barcode bases for each sample[28]. No barcode was added to the forward primer. Hence the PCR products were flanked by a 40 bp fusion primer/multiplex identifier sequence at the reverse end and a 30 bp fusion primer at the forward end. PCR cycling conditions were as follows: 2 minutes at 94°C; 20 cycles of 30 seconds at 94°C, 30 seconds at 53°C, 120 seconds at 68°C. Following confirmation of adequate and appropriately sized product, the quadruplicate reactions were pooled and ethanol precipitated prior to purification as per the recommended AMPure purification method for 454 sequencing. The PCR products were then sequenced with the Roche 454 Titanium sequencing platform using the Lib-L kit (Wellcome Trust Sanger Institute, Cambridgeshire, UK). The sequence data are available from the European Nucleotide Archive under Study Accession Numbers ERP004371 and ERP004372, and Sample Accession Numbers ERS373486 and ERS373498. The relevant barcode information for each of the samples is shown in Table S1.

Quantitative PCR

Quantitative real-time PCR was performed as described previously[29]. Briefly, standard curves consisted of ten-fold dilution series of amplified bacterial 16S rRNA genes from reference strains. Samples were amplified with universal primers against total bacteria and specific primers against *Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Enterobacteriaceae* (Table 1). The abundance of 16S rRNA gene copies was determined from standard curves and specific bacterial groups were expressed as a

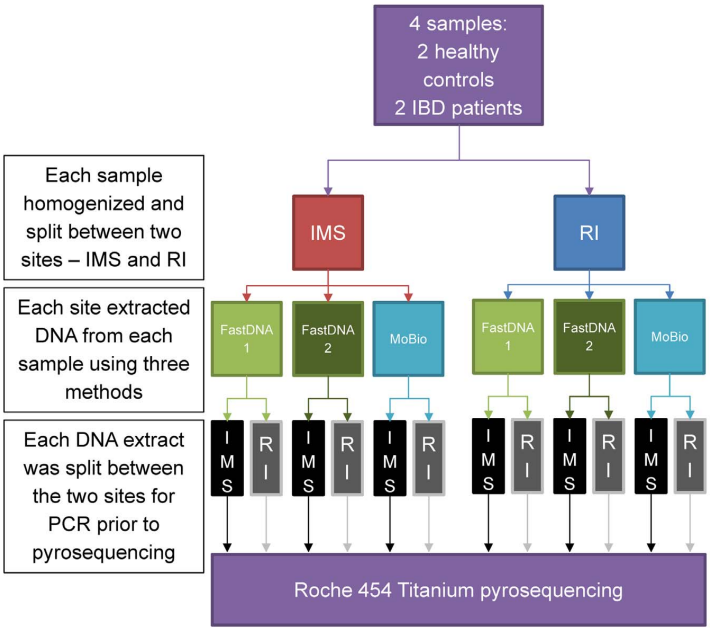


Figure 1. Study protocol. IMS: Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen; RI: Rowett Institute of Nutrition and Health, University of Aberdeen, Bucksburn, Aberdeen.
doi:10.1371/journal.pone.0088982.g001

percentage of total bacteria determined by universal primers. 5 ng of DNA was used per reaction. The same DNA concentration was used for all runs, including universal primer runs which were used to normalize specific bacterial groups against total bacteria, to minimize errors due to any inhibitory substances in the samples. The detection limit was determined with negative controls containing only herring sperm DNA.

Bioinformatic and Statistical Analysis

Analysis of sequence data was carried out using the Mothur software package[30]. Initially, the "trim.seqs" function was used to filter reads for quality by truncating them once average quality scores dropped below 35 across a rolling window of 50 bases. In addition all reads with any mismatches to the primer or barcode sequences, plus reads with ambiguous bases (i.e. "N"s) or with homopolymeric stretches of longer than 8 bases were removed. Read length following this step ranged from 336 to 351 bp. Chimeras were then checked for and removed using Perseus software[31], as implemented in Mothur. The sequences were then aligned to the reference SILVA database provided in Mothur, a distance matrix generated, and then clustered into operational taxonomic units (OTUs) at 97% similarity using the average neighbor setting in Mothur. Each OTU was assigned a taxonomic classification at all levels from Phylum to Genus using

Table 1. qPCR primers used.

Bacterial family	Primer name	Primer sequence	Reference
All bacteria	UniF	GTGSTGCAYGGYYGTCGTCA	[38]
	UniR	ACGTCRTCCMCNCCTTCCTC	
Bacteroidaceae	Bac303F	GAAGGTCCCCCACATTG	
	Bfr-Fmrev	CGCKACTTGGCTGGTTCAG	
Ruminococcaceae	Clep866mF	TTAACACAATAAGTWATCCACCTGG	
	Clept1240mR	ACCTTCCTCCGTTTTGTCAAC	
Lachnospiraceae	Erec482F	CGGTACCTGACTAAGAAGC	
	Erec870R	AGTTTYATTCTTGCGAACG	
Enterobacteriaceae	EnterobactDmod2F	GACCTCGCGAGAGCA	[29]
	Enter1432mod	CCTACTTCTTTTGCAACCCA	

doi:10.1371/journal.pone.0088982.t001

the reference Ribosomal Database Project database (RDP) provided in Mothur with the *Gemmiger/Subdoligranulum* classification error corrected. Jaccard and Yue and Clayton distance matrices were calculated using the vegan package in R[32]. Dendrograms were generated using Ward clustering, and then visualized using the iTOL web package[33].

Comparisons in DNA yield were performed using Mann Whitney U testing. Linear modelling was used to assess the relative contribution of patient, DNA extraction method and extraction site to the measured proportions of different bacterial families. Log-transformed data was used to permit analysis of the fold change. A model was constructed for each bacterial family using the donor source, extraction method and extraction site as covariates. Bacterial families were reported where at least one sample had an abundance of 5% or more. For each family, samples were only included from participants with at least 0.5% abundance for that bacterial family in one or more of their samples. Modelling was also done in a similar manner using individual OTUs. When using linear modelling at the OTU level, Holm's method was used to correct for multiple testing. Correlation between pyrosequencing and qPCR data was done using Pearsons's correlation coefficient. Analysis was performed using R 2.15.2 (R Statistical Foundation, Vienna, Austria).

Results

DNA yields were significantly higher with either method of the FastDNA kit than with the MoBio kit, with median DNA concentrations of 476 ng/μL (interquartile range [IQR] 290–519) for FastDNA method 1, 453 ng/μL (IQR 228–689) for FastDNA method 2 and 22 ng/μL (IQR 9-36) for the MoBio method ($p<0.001$ for both comparisons, Figure 2). There was no significant difference in yield between the two methods of the FastDNA kit ($p = 0.798$).

Compositional analysis indicated a higher proportion of *Enterobacteriaceae* and *Sutterellaceae*, and lower *Ruminococcaceae*, in the samples from the two IBD patients compared with the two healthy controls, regardless of the extraction method or laboratory. Although this study was clearly not powered to differentiate

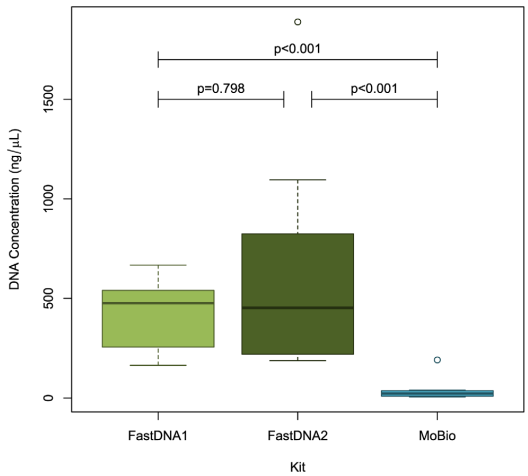


Figure 2. Comparison of DNA yields between extraction methods.
doi:10.1371/journal.pone.0088982.g002

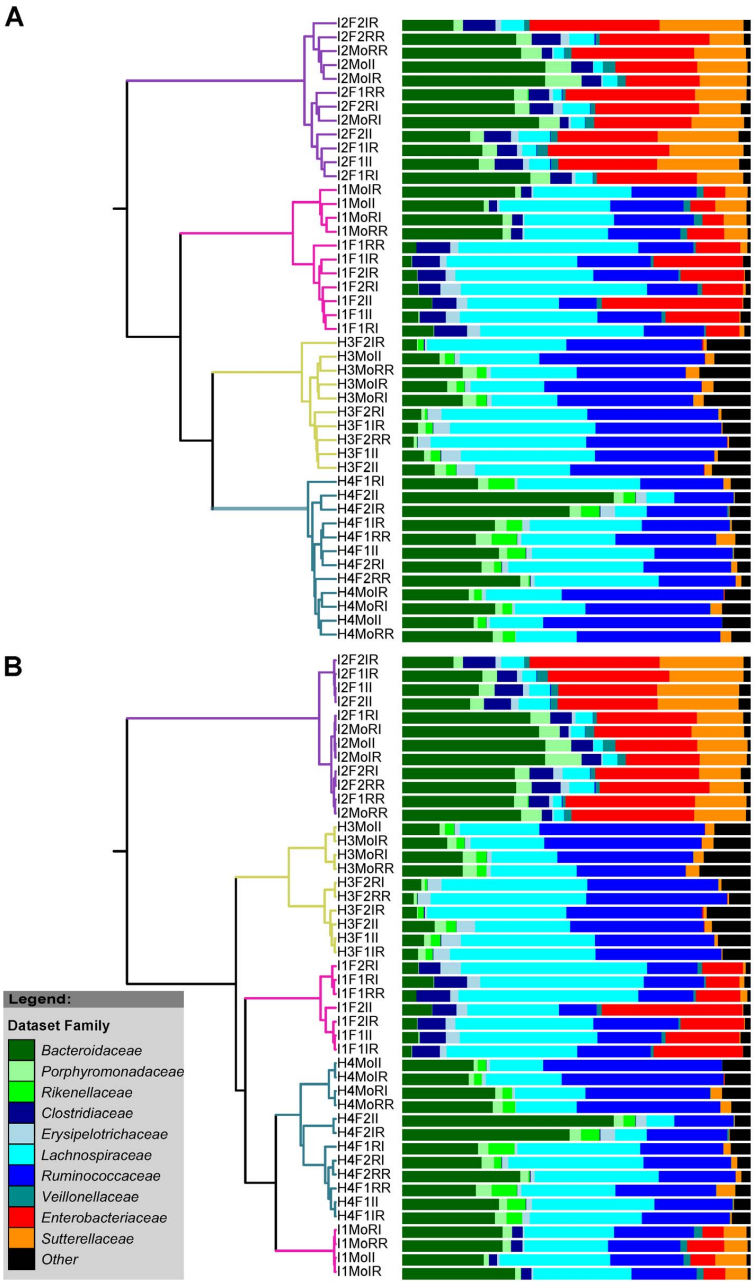
between IBD cases and controls the higher observed proportions of Proteobacteria in cases, particularly case I2, is consistent with patterns described previously in IBD[6,7].

Clustering of the microbiota composition derived from the sequence data for these samples was carried out using both the Jaccard and the Yue and Clayton calculators. The Jaccard calculator is used to describe overlap in community membership between different samples and ignores the proportional abundance of each OTU while, in contrast, the Yue and Clayton calculator takes the proportional abundance of each OTU into account when comparing community similarities. Jaccard-based calculations revealed a clear clustering of samples primarily by subject of origin (Figure 3a). This is as expected given the well-known inter-individual variation in microbiota composition between individuals[34]. Within individuals, however, the MoBio-processed samples tended to cluster together, separately to those processed using the FastDNA kit, indicating that, although there were overall similarities in the range of organisms that were identified using the two DNA extraction kits, there is some bias associated with the use of each kit. More serious repercussions of using different DNA extraction kits were observed when using the Yue and Clayton distance metric, where dominant organisms can have more impact on clustering patterns. The MoBio-processed samples of subject I1 clustered with the samples from subject H4 rather than with the FastDNA-processed samples from patient I1, presumably as a result of elevated *Bacteroides* and lower *Lachnospiraceae* proportional abundances in the MoBio extractions compared to the FastDNA extractions (Figure 3b). This demonstrates that biases introduced by DNA extraction methodology can over-ride the real underlying patterns of community structure driven by inter-individual variation.

Linear modelling of the family level data for the top nine families represented in the pyrosequencing data is shown in Table 2 (range of abundances in table S2). Significant differences were identified between the FastDNA and MoBio kits, with relatively higher *Bacteroidaceae*, *Ruminococcaceae* and *Porphyromonadaceae*, and lower *Enterobacteriaceae*, *Lachnospiraceae*, *Clostridiaceae* and *Erysipelotrichaceae* following extraction with the MoBio kit. There was a significant difference identified between the two methods of the FastDNA kit in just one family, the *Rikenellaceae*. The extraction site made a significant difference only for *Sutterellaceae*, with the observed differences being driven by an increase in one OTU in samples from patients H4 and I1 when they had been extracted at RINH (Using FastDNA methods in patient H4, relative abundance 0.24% (95% confidence interval 0.11-0.38%) at IMS and 2.64% (0.85-4.44%) at RINH.) The site at which the amplification PCR was performed made no significant difference for any of the bacterial families analyzed, and was therefore excluded from the models.

At the OTU level, 18 OTUs were significantly different between the MoBio kit and the FastDNA kit after correction for multiple testing (Table 3). Of these, 10 were from the *Lachnospiraceae* family and 8 of these 10 were relatively under-represented in the MoBio processed samples, in some cases with a complete absence of the OTU in the MoBio samples.

Correlation between pyrosequencing and qPCR data was generally good (Figure 4), with R^2 values of 0.81, 0.86 and 0.94 for *Ruminococcaceae*, *Bacteroidaceae* and *Enterobacteriaceae* respectively. However, the correlation was less good for the *Lachnospiracaeae*, with an R^2 value of 0.42. Linear modelling revealed similar differences to that seen in the pyrosequencing data, although the differences related to extraction method were only significant for *Ruminococcaceae* and *Enterobacteriaceae* (Tables 4, S3).



Key to sample names

Patient or control	Extraction method	Site of DNA extraction	Site of PCR
I1 I2 (IBD patients) H3 H4 (healthy controls) Clades within the tree are coloured acccording to donor	F1, F2 (FastDNA methods 1 and 2) Mo (MoBio PowerSoil)	I (Institute of Medical Sciences) R (Rowett Institute)	

Figure 3. Dendrogram of the representation of bacterial families derived from 16S rRNA gene sequences within each sample clustered by Jaccard (A) and Yue and Clayton (B) distances.
doi:10.1371/journal.pone.0088982.g003

Discussion

With the recognition that cultured bacteria cover only a small proportion of gut microbial diversity[35], a number of molecular techniques have been developed to describe and quantify the gut microbiota, from qualitative gel-based methods to full metagenomic sequencing[5,36]. Almost all of these techniques require extraction of DNA from fecal or mucosal samples as a first step, and differences at this point will influence downstream results. The importance of this will be amplified if, for example, cases and controls are processed in a different manner.

This study highlights important differences in the performance of two commercially available kits for DNA extraction from fecal samples. Significantly lower DNA yields were seen with the MoBio kit than the FastDNA kit. This is consistent with results published previously[19]. More importantly, there were significant differences in the relative abundance of bacteria measured at both the family and OTU level. There is no gold standard to which these data can be compared, and so it is impossible to say which technique yields results closer to the true profile of the samples. However, the lower yield of the MoBio kit, and reduced proportional abundance of the *Lachnospiraceae* family of Firmicutes, suggests that this kit may not be stringent enough for optimal lysing of some Gram-positive organisms. Regardless, these differences are such that it is important to stipulate that all samples in a particular experiment should be extracted using the same technique. This is of particular importance with multicenter studies. Moreover, it should prompt researchers to exercise caution when comparing datasets from different studies. Indeed, if DNA has been extracted using different kits then studies should not be considered cross-comparable. Of note, a recent meta-analysis found that samples from studies of fecal microbiota within Western populations clustered by study, suggesting that systematic bias was introduced by factors such as DNA extraction technique[37].

The importance of the observed differences will depend on the analysis techniques used. However, whenever a relative quantification technique is used, the results for even a single organism will be influenced by the effects of the extraction technique on the total number of bacteria isolated relative to that specific species. The methods for both kits used here involved physical disruption by bead-beating. Methods that rely on enzymatic treatment without physical disruption have been shown previously to give biased recovery, with reduced recovery of Gram-positive organisms and artificially elevated levels of Gram negatives, presumably because these are more easily lysed[18,22].

A smaller effect was observed of the extraction site on relative abundance, with only *Sutterellaceae* reaching statistical significance. This may reflect a difference in operator, equipment or laboratory environment. To minimize the influence of differences between laboratories, centralization of DNA extraction for an experiment would be preferred. The technique described here includes only minimal processing after sample collection prior to interim storage at -80°C. This allows for collection sites to collate a number of samples in -80°C storage prior to shipment to a central facility for DNA extraction and downstream analysis.

The qPCR data in general correlated well with that from pyrosequencing with the exception of *Lachnospiracaeae*. This can be partially explained by differences between the range of organisms that were targeted by the qPCR primers and those that were classified as *Lachnospiracaeae* in the pyrosequencing data, although 78% of OTUs and 89% of sequences labelled as *Lachnospiracaeae* were estimated *in silico* to be targeted by the qPCR primer set used.

Ariefdjohan et al. previously assessed the effect of DNA extraction method on the measured bacterial composition of stool using denaturing gradient gel electrophoresis (DGGE)[19]. This study demonstrated variability in bacterial community between fecal samples extracted with QIAamp DNA, MoBio Ultra Clean Fecal DNA and FastDNA SPIN kits, and noted that both the MoBio and Qiagen kits were not able to extract DNA from all the bacteria in the specimen. More recently, Claassen et al. used DGGE, terminal restriction fragment length polymorphism (T-RFLP) and qPCR to compare fecal samples extracted using kits from Qiagen, ZymoResearch and MoBio and found few

Table 2. Linear modelling of family-level pyrosequencing data.

Bacterial Family	Kit				Extraction Site		
	FastDNA 2 fold change	p	MoBio fold change	P	RINH fold change	p	Patients included
Lachnospiraceae	0.96 (0.74–1.25)	0.775	0.63 (0.49–0.81)	0.001	1.17 (0.95–1.44)	0.160	H3,H4,I1,I2
Bacteroidaceae	1.13 (0.79–1.63)	0.501	2.13 (1.49–3.05)	<0.001	1.09 (0.81–1.46)	0.561	H3,H4,I1,I2
Ruminococcaceae	0.94 (0.79–1.13)	0.524	1.32 (1.11–1.58)	0.005	0.95 (0.82–1.10)	0.516	H3,H4,I1
Enterobacteriaceae	1.08 (0.74–1.57)	0.695	0.61 (0.43–0.88)	0.016	0.85 (0.63–1.15)	0.311	I1,I2
Sutterellaceae	0.77 (0.18–3.37)	0.735	1.11 (0.26–4.69)	0.892	3.84 (1.18–12.46)	0.031	H3,H4,I1,I2
Clostridiaceae	1.00 (0.77–1.30)	0.976	0.46 (0.36–0.59)	<0.001	0.88 (0.71–1.08)	0.243	I1,I2
Porphyromonadaceae	1.46 (0.41–5.19)	0.560	4.03 (1.16–14.01)	0.035	0.70 (0.26–1.94)	0.502	H3,H4,I1,I2
Erysipelotrichaceae	1.21 (0.81–1.81)	0.361	0.32 (0.21–0.47)	<0.001	0.88 (0.64–1.22)	0.445	H3,H4,I1,I2
Rikenellaceae	0.35 (0.16–0.76)	0.016	0.72 (0.33–1.56)	0.418	0.65 (0.35–1.19)	0.181	H3,H4

RINH: Rowett Institute of Nutrition and Health.
Participants were excluded if all data points for that bacterial family were < 0.5%. Reference sample was from participant H3 using FastDNA method 1 and extracted at the Institute of Medical Sciences. Differences are shown as fold change with 95% confidence intervals.
doi:10.1371/journal.pone.0088982.t002

Table 3. Multiple linear modelling after correction for multiple testing shows OTUs with significantly different relative abundance after extraction with the MoBio kit.

Genus	Family	Order	Class	Phylum	Fold change	p	Corrected p	Patients included
Eggerthella	*Coriobacteriaceae*	Coriobacteriales	Actinobacteria	Actinobacteria	0.00 (0.00–0.00)	5.09×10^{-9}	5.55×10^{-7}	I1
Blautia	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	0.00 (0.00–0.01)	1.70×10^{-7}	1.83×10^{-5}	H3,H4,I1
Blautia	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	0.01 (0.00–0.04)	2.01×10^{-7}	2.15×10^{-5}	H3,H4,I1
Bacteroides	*Bacteroidaceae*	Bacteroidales	Bacteroidia	Bacteroidetes	2.60 (1.92–3.52)	3.26×10^{-7}	3.46×10^{-5}	H3,H4,I1,I2
Clostridium sensu stricto	*Clostridiaceae*	Clostridiales	Clostridia	Firmicutes	0.36 (0.30–0.43)	3.91×10^{-6}	0.0004	I1
Blautia	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	0.01 (0.00–0.05)	1.97×10^{-5}	0.0020	H3,H4,I1
unclassified	*Ruminococcaceae*	Clostridiales	Clostridia	Firmicutes	0.17 (0.12–0.24)	3.13×10^{-5}	0.0032	H3
unclassified	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	0.00 (0.00–0.01)	3.49×10^{-5}	0.0036	H4,I1
Anaerostipes	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	0.10 (0.04–0.23)	5.23×10^{-5}	0.0053	H3,H4
Escherichia Shigella	*Enterobacteriaceae*	Enterobacteriales	Gamma-proteobacteria	Proteobacteria	0.41 (0.28–0.59)	1.36×10^{-4}	0.0136	I1,I2
unclassified	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	5.05 (2.55–9.97)	2.24×10^{-4}	0.0222	H3,I1
Ruminococcus	*Ruminococcaceae*	Clostridiales	Clostridia	Firmicutes	0.48 (0.34–0.68)	0.0002	0.0232	H3,H4,I1
unclassified	*Lachnospiraceae*	Clostridiales	Clostridia	Firmicutes	2.50 (1.92–3.26)	0.0003	0.0250	H3
unclassified	*Ruminococcaceae*	Clostridiales	Clostridia	Firmicutes	3.19 (2.26–4.50)	0.0003	0.0298	H3
Bacteroides	Bacteroidaceae	Bacteroidales	Bacteroidia	Bacteroidetes	2.03 (1.42–2.89)	0.0004	0.0342	H3,H4,I1,I2
Dorea	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes	0.00 (0.00–0.06)	0.0004	0.0343	H3,H4,I1
unclassified	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes	0.00 (0.00–0.00)	0.0004	0.0358	I1
Dorea	Lachnospiraceae	Clostridiales	Clostridia	Firmicutes	0.06 (0.01–0.25)	0.0004	0.0381	H3,H4,I1,I2

Samples were excluded if all data points for that bacterial family were <0.5%. Reference sample was from patient H3 using either FastDNA method and extracted at the Institute of Medical Sciences. Differences are shown as fold change with 95% confidence intervals.
doi:10.1371/journal.pone.0088982.t003

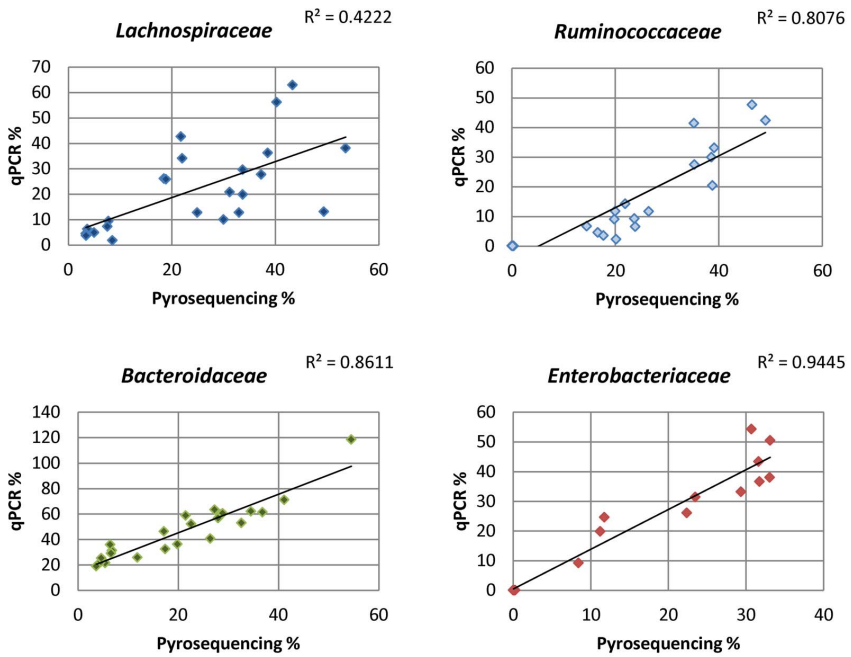


Figure 4. Correlation between pyrosequencing and qPCR data.
doi:10.1371/journal.pone.0088982.g004

Table 4. Linear modelling of qPCR data.

Bacterial Family	Kit				Extraction Site		
	FastDNA 2 fold changep		MoBio fold change	P	RINH fold change	p	Patients included
Lachnospiraceae	0.75 (0.48–1.17)	0.209	0.74 (0.48–1.16)	0.199	1.35 (0.94–1.94)	0.107	H3,H4,I1,I2
Bacteroidaceae	1.19 (0.94–1.51)	0.147	1.25 (0.99–1.57)	0.066	0.98 (0.81–1.18)	0.822	H3,H4,I1,I2
Ruminococcaceae	0.69 (0.47–1.01)	0.070	2.32 (1.58–3.39)	<0.001	1.26 (0.92–1.71)	0.157	H3,H4,I1
Enterobacteriaceae	1.28 (0.97–1.69)	0.102	0.65 (0.48–0.87)	0.011	0.91 (0.72–1.15)	0.436	I1,I2

RINH: Rowett Institute of Nutrition and Health.
Participants were excluded if all data points for that bacterial family were <0.5%. Reference sample was from participant H3 using FastDNA method 1 and extracted at the Institute of Medical Sciences. Differences are shown as fold change with 95% confidence intervals.
doi:10.1371/journal.pone.0088982.t004

significant differences[20]. In contrast, the previous study by Wu *et al.* which assessed the effect of extraction methods on 16S rRNA pyrosequencing demonstrated increased yield of Firmicutes when a hot phenol bead-beating method or the PSP kit were used. The present study helps bring further clarity to this important issue with next generation sequencing permitting a more detailed exploration of the differences between samples extracted with different methods.

This study is somewhat limited by its relatively small sample size, with fecal samples obtained from only four individuals. There were a small number of outliers; samples H4F2AA and H4F2AR had much higher relative abundance of *Bacteroidaceae*. In addition, the data obtained here from both pyrosequencing and qPCR estimate relative abundance rather than absolute numbers and focus on the dominant groups within the microbiota.

Conclusions

This study demonstrates important differences in the yield and relative abundance of key bacterial families for kits used to isolate bacterial DNA from stool. This highlights the importance of ensuring that all samples to be analyzed together are prepared with the same DNA extraction method, and the need for caution when comparing studies that have used different methods.

Supporting Information

Table S1 Barcodes used for each sample included in the study, and the respective ENA-deposited dataset that they can be recovered from.
(XLSX)

Table S2 Relative abundances of the top nine bacterial families measured for each individual.
(DOCX)

Table S3 Relative abundances of the bacterial families measured by quantitative PCR for each individual.
(DOCX)

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References

1. The Human Microbiome Project (2012) Structure, function and diversity of the healthy human microbiome. Nature 486: 207–214. doi:10.1038/nature11234.
2. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, et al. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59–65. doi:10.1038/nature08821.
3. Whitman WB, Coleman DC, Wiebe WJ (1998) Perspective Prokaryotes: The unseen majority. 95: 6578–6583.
4. Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124: 837–848. doi:10.1016/j.cell.2006.02.017.
5. Sekirov I, Russell SL, Antunes LCM, Finlay BB (2010) Gut microbiota in health and disease. Physiol Rev 90: 859–904. doi:10.1152/physrev.00045.2009.
6. Sartor RB (2006) Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 3: 390–407. doi:10.1038/ncpgasthep0528.
7. Manichanh C, Borruel N, Casellas F, Guarner F (2012) The gut microbiota in IBD. Nat Rev Gastroenterol Hepatol 9: 599–608. doi:10.1038/nrgastro.2012.152.
8. Simrén M, Barbara G, Flint HJ, Spiegel BMR, Spiller RC, et al. (2013) Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 62: 159–176. doi:10.1136/gutjnl-2012-302167.
9. Musso G, Gambino R, Cassader M (2011) Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. Annu Rev Med 62: 361–380. doi:10.1146/annurev-med-012510-175505.
10. Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. J Nutr 134: 465–472.
11. Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59: 143–169.
12. Persson S, de Boer RF, Kooistra-Smid AMD, Olsen KEP (2011) Five commercial DNA extraction systems tested and compared on a stool sample collection. Diagn Microbiol Infect Dis 69: 240–244. doi:10.1016/j.diagmicrobio.2010.09.023.
13. McOrist AL, Jackson M, Bird AR (2002) A comparison of five methods for extraction of bacterial DNA from human faecal samples. J Microbiol Methods 50: 131–139. doi:10.1016/S0167-7012(02)00018-0.
14. Nechvatal JM, Ram JL, Basson MD, Namprachan P, Niec SR, et al. (2008) Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. J Microbiol Methods 72: 124–132. doi:10.1016/j.mimet.2007.11.007.
15. Maukonen J, Simões C, Saarela M (2012) The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. FEMS Microbiol Ecol 79: 697–708. doi:10.1111/j.1574–6941.2011.01257.x.
16. Smith B, Li N, Andersen AS, Slotved HC, Krogfelt KA (2011) Optimising bacterial DNA extraction from faecal samples: comparison of three methods. Open Microbiol J 5: 14–17. doi:10.2174/1874285801105010014.
17. Yu Z, Morrison M (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36: 808–812.
18. Zoetendal EG, Ben-Amor K, Akkermans AD, Abee T, de Vos WM (2001) DNA isolation protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract. Syst Appl Microbiol 24: 405–410. doi:10.1078/0723-2020-00060.
19. Ariefdjohan MW, Savaiano DA, Nakatsu CH (2010) Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutr J 9: 23. doi:10.1186/1475-2891-9-23.
20. Claassen S, du Toit E, Kaba M, Moodley C, Zar HJ, et al. (2013) A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. J Microbiol Methods 94: 103–110. doi:10.1016/j.mimet.2013.05.008.
21. Salonen A, Nikkilä J, Jalanka-Tuovinen J, Immonen O, Rajilić-Stojanović M, et al. (2010) Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J Microbiol Methods 81: 127–134. doi:10.1016/j.mimet.2010.02.007.
22. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ (2012) Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One 7: e33865. doi:10.1371/journal.pone.0033865.
23. Milani C, Hevia A, Foroni E, Duranti S, Turroni F, et al. (2013) Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. PLoS One 8: e68739. doi:10.1371/journal.pone.0068739.
24. Wu GD, Lewis JD, Hoffmann C, Chen Y-Y, Knight R, et al. (2010) Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol 10: 206. doi:10.1186/1471-2180-10-206.
25. Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N (2010) Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol Lett 307: 80–86. doi:10.1111/j.1574-6968.2010.01965.x.
26. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, et al. (2011) Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J 5: 220–230. doi:10.1038/ismej.2010.118.
27. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, et al. (2009) Bacterial community variation in human body habitats across space and time. Science 326: 1694–1697. doi:10.1126/science.1177486.
28. Fierer N, Hamady M, Lauber CL, Knight R (2008) The influence of sex, handedness, and washing on the diversity of hand surface bacteria. Proc Natl Acad Sci U S A 105: 17994–17999. doi:10.1073/pnas.0807920105.
29. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, et al. (2012) Microbiota of De-Novo Pediatric IBD: Increased *Faecalibacterium Prausnitzii* and Reduced Bacterial Diversity in Crohn's But Not in Ulcerative Colitis. Am J Gastroenterol: 1–10. doi:10.1038/ajg.2012.335.
30. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541. doi:10.1128/AEM.01541-09.
31. Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ (2011) Removing noise from pyrosequenced amplicons. BMC Bioinformatics 12: 38. doi:10.1186/1471-2105-12-38.
32. Yue JC, Clayton MK, Lin FC (2001) A nonparametric estimator of species overlap. Biometrics 57: 743–749. doi:10.1111/j.0006-341X.2001.00743.x.
33. Letunic I, Bork P (2011) Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Res 39: W475–8. doi:10.1093/nar/gkr201.
34. Flint HJ, Scott KP, Louis P, Duncan SH (2012) The role of the gut microbiota in nutrition and health. Nat Rev Gastroenterol Hepatol 9: 577–589. doi:10.1038/nrgastro.2012.156.
35. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. (2005) Diversity of the human intestinal microbial flora. Science 308: 1635–1638. doi:10.1126/science.1110591.
36. Lawley B, Tannock GW (2012) Nucleic acid-based methods to assess the composition and function of the bowel microbiota. Gastroenterol Clin North Am 41: 855–868. doi:10.1016/j.gtc.2012.08.010.
37. Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, et al. (2013) Meta-analyses of studies of the human microbiota. Genome Res 23: 1704–1714. doi:10.1101/gr.151803.112.
38. Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, et al. (2009) Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. Br J Nutr 101: 541–550. doi:10.1017/S0007114508019880.

Supplementary tables

Table S1 - Barcodes used for each sample included in the study, and the respective ENA-deposited dataset that they can be recovered from.
Note that some samples had low sequence counts in the first run and so were re-sequenced. Data from the second sequencing run are found in the smaller second portion of the table below.

Sample Name	Barcode	ENA Study Accession Number	ENA Sample Accession Number
I1F1II	AACGCACGCTAG	ERP004371	ERS373486
I1F2II	AACTCGTCGATG	ERP004371	ERS373486
I2F1II	AACTGTGCGTAC	ERP004371	ERS373486
I2F2II	AAGAGATGTCGA	ERP004371	ERS373486
I1MoII	AAGCTGCAGTCG	ERP004371	ERS373486
I2MoII	AATCAGTCTCGT	ERP004371	ERS373486
H3F1II	AATCGTGACTCG	ERP004371	ERS373486
H3F2II	ACACACTATGGC	ERP004371	ERS373486
H4F1II	ACACATGTCTAC	ERP004371	ERS373486
H4F2II	ACACGAGCCACA	ERP004371	ERS373486
H3MoII	ACACGGTGTCTA	ERP004371	ERS373486
H4MoII	ACACTAGATCCG	ERP004371	ERS373486
I1F1RI	ACACTGTTCATG	ERP004371	ERS373486
I1F2RI	ACAGACCACTCA	ERP004371	ERS373486
I2F1RI	ACAGAGTCGGCT	ERP004371	ERS373486
I2F2RI	ACAGCAGTGGTC	ERP004371	ERS373486
I1MoRI	ACAGCTAGCTTG	ERP004371	ERS373486
I2MoRI	ACAGTGCTTCAT	ERP004371	ERS373486
H3F1RI	ACAGTTGCGCGA	ERP004371	ERS373486
H3F2RI	ACATCACTTAGC	ERP004371	ERS373486
H4F1RI	ACATGATCGTTC	ERP004371	ERS373486
H4F2RI	ACATGTCACGTG	ERP004371	ERS373486
H3MoRI	ACATTCAGCGCA	ERP004371	ERS373486
H4MoRI	ACCACATACATC	ERP004371	ERS373486
I1F1IR	AGATGTTCTGCT	ERP004371	ERS373486
I1F2IR	AGCACACCTACA	ERP004371	ERS373486
I2F1IR	AGCACGAGCCTA	ERP004371	ERS373486
I2F2IR	AGCAGCACTTGT	ERP004371	ERS373486
H3F1IR	AGCAGTCGCGAT	ERP004371	ERS373486
H3F2IR	AGCATATGAGAG	ERP004371	ERS373486
H4F1IR	AGCCATACTGAC	ERP004371	ERS373486
H4F2IR	AGCGACTGTGCA	ERP004371	ERS373486
I1MoIR	AGCGAGCTATCT	ERP004371	ERS373486
I2MoIR	AGCGCTGATGTG	ERP004371	ERS373486
H3MoIR	AGCGTAGGTCGT	ERP004371	ERS373486

H4MoIR	AGCTATCCACGA	ERP004371	ERS373486
I1F1RR	AGCTCCATACAG	ERP004371	ERS373486
I1F2RR	AGCTCTCAGAGG	ERP004371	ERS373486
I2F1RR	AGCTGACTAGTC	ERP004371	ERS373486
I2F2RR	AGCTTGACAGCT	ERP004371	ERS373486
H3F1RR	AGGACGCACTGT	ERP004371	ERS373486
H3F2RR	AGGCTACACGAC	ERP004371	ERS373486
H4F1RR	AGGTGTGATCGC	ERP004371	ERS373486
H4F2RR	AGTACGCTCGAG	ERP004371	ERS373486
I1MoRR	AGTACTGCAGGC	ERP004371	ERS373486
I2MoRR	AGTAGTATCCTC	ERP004371	ERS373486
H3MoRR	AGTCACATCACT	ERP004371	ERS373486
H4MoRR	AGTCCATAGCTG	ERP004371	ERS373486
I1F2IR	AGCACACCTACA	ERP004372	ERS373498
H4F2IR	AGCGACTGTGCA	ERP004372	ERS373498
I1F2RR	AGCTCTCAGAGG	ERP004372	ERS373498
H3F1RR	AGGACGCACTGT	ERP004372	ERS373498
H3F2RR	AGGCTACACGAC	ERP004372	ERS373498
I1F2II	AACTCGTCGATG	ERP004372	ERS373498
H4F2II	ACACGAGCCACA	ERP004372	ERS373498
I1F2RI	ACAGACCACTCA	ERP004372	ERS373498
H3F1RI	ACAGTTGCGCGA	ERP004372	ERS373498
H3F2RI	ACATCACTTAGC	ERP004372	ERS373498

Table S2: Relative abundances of the top nine bacterial families measured for each individual

Bacterial Family	Relative abundance median (range [%])				
	H3	H4	I1	I2	
Lachnospiraceae	33.00 (18.93-44.72)	24.41 (7.99-38.91)	37.53 (24.10-53.59)	4.49 (2.50-8.95)	
Bacteroidaceae	7.82 (3.36-17.46)	26.37 (19.18-60.88)	8.54 (2.69-32.45)	32.61 (14.79-41.21)	
Ruminococcaceae	38.75 (31.33-47.65)	25.21 (17.06-51.47)	18.77 (10.86-24.51)	0.11 (0.00-0.46)	
Enterobacteriaceae	0.05 (0.00-0.33)	0.00 (0.00-0.18)	11.73 (6.12-40.43)	29.34 (21.23-37.40)	
Sutterellaceae	1.58 (0.30-3.86)	1.05 (0.00-5.50)	1.40 (0.07-9.06)	14.83 (9.90-24.16)	
Clostridiaceae	0.08 (0.03-0.33)	0.09 (0.00-0.22)	7.04 (2.36-9.66)	6.24 (2.57-9.33)	
Porphyromonadaceae	2.05 (0.33-4.15)	2.86 (1.17-4.58)	0.16 (0.00-2.76)	4.52 (2.71-10.50)	
Erysipelotrichaceae	2.68 (0.65-5.67)	1.18 (0.25-4.31)	2.41 (0.48-5.87)	1.31 (0.13-2.66)	
Rikenellaceae	2.54 (0.18-3.00)	3.49 (0.37-7.56)	0.00 (0.00-0.09)	0.00 (0.00-0.34)	

Table S3: Relative abundances of the bacterial families measured by quantitative PCR for each individual

Bacterial Family	Relative abundance median (range [%])				
	H3	H4	I1	I2	
Lachnospiraceae	42.69 (29.64-63.00)	23.33 (1.83-27.76)	12.78 (10.06-38.12)	5.59 (3.75-9.43)	
Bacteroidaceae	25.74 (21.45-32.36)	59.21 (36.26-118.38)	35.85 (18.90-60.49)	57.19 (46.37-71.11)	
Ruminococcaceae	33.11 (20.44-47.67)	10.49 (2.29-42.40)	9.06 (3.55-14.27)	0.05 (0.05-0.07)	
Enterobacteriaceae	0.02 (0.01-0.04)	0.01 (0.00-0.01)	24.58 (9.19-33.23)	40.73 (26.08-54.22)	

4.3 Discussion of paper

This paper helped us determine the most appropriate experimental approach to use in the experiment detailed in the next chapter. It also provided opportunities to develop some of the techniques required for this larger experiment. Since its publication in 2014, it has been cited 183 times, reflecting the importance of such methodological work.

The choice of DNA extraction method is one the most important causes of technical experimental variation in studies of faecal microbiota. [128]The importance of bead beating, as used in the kits here, has been highlighted in terms of improving yield of difficult-to-lyse organisms.[129–131] Subsequent studies have compared the PowerSoil kit used here with other alternatives. Some, such as a 2019 paper by Videnska *et al.* again found that the PowerSoil kit was superior in terms of lysis of specific taxa.[129] Other groups had less success with the kit, although this was influenced by the lack of use of the specialised equipment recommended.[132] Overall, there is not a consensus on a single method to be used for all microbiome experiments. In general, techniques that include mechanical lysis, typically bead beating, are preferred, but the most important aspect is to optimise DNA yield and quality without introducing bias with respect to specific taxa.[131]

Other than differences in extraction method, other important aspects that can influence study findings include the choice of primers and region of 16S RRNA, the choice of sequencing platform and the analytical tools used to process the data.[131] The large number of permutations of these various choices made for experimental design mean that it is important to use consistent methodology within a single study and to compare across studies with caution. Reassuringly, as seen in the paper here there is less impact of laboratory location in the results seen of microbiome experiments.

5 The Impact of NOD2 Variants on Fecal Microbiota in Crohn's Disease and Controls Without Gastrointestinal Disease

5.1 Introduction to paper

Research to date has indicated that Crohn's disease is the result of a complex interaction between genes, the environment and the gut microbiota.[19] *NOD2* was the first-described genetic association with Crohn's disease and remains one of the most important genetic determinants for the development of disease,[13] as well as for predicting ileal location.[18] *NOD2* is a pattern recognition receptor which detects the presence of muramyl dipeptide and triggers innate immune responses. It is therefore plausible that disease-associated variation in *NOD2* might lead to alterations in the gut microbiota. A small number of studies had assessed the gene-microbiota interactions for *NOD2* but had limited numbers of individuals homozygous for *NOD2* mutations since, even in Crohn's disease, the prevalence of homozygotes or compound heterozygotes is relatively low.

We took advantage of the well-phenotyped and genotyped UK IBD genetics consortium cohort. This cohort of patients has been assembled by a collaborative group of clinicians and scientists from around the UK, with a large contribution from Edinburgh. Using available genetic and phenotype data, I was able to select cases of Crohn's disease carrying two Crohn's-associated *NOD2* variants as well as matched patients who were wild type for *NOD2*. We recruited genotype-matched controls without gastrointestinal disease from the Cambridge BioResource, as well as household members of the patients with Crohn's disease.

5.2 Contributions

I designed the study, working with Georgina Hold, Charlie Lees and Alan Walker. I co-ordinated sample and data collection from the four clinical sites and Cambridge BioResource, under the supervision of Charlie Lees. I collected the Edinburgh samples myself (including visits to patients' homes), while samples from Newcastle, Cambridge and Norwich were collected by researchers based at those sites. I performed the DNA extraction from the samples under the supervision of Susan Berry and Georgina Hold. I also undertook protein extraction for calprotectin measurement under the supervision of the Western General biochemistry/GI pathology laboratory. Chris Probert measured the volatile organic compounds using gas chromatography mass spectrometry, while Alan Walker and Julian Parker generated the 16S rRNA sequence data. I undertook the analysis of the clinical, sequence and VOC data under supervision from Georgina Hold and Charlie Lees. I wrote the paper with input from Georgina Hold and Charlie Lees.

Published as: Kennedy NA, Lamb CA, Berry SH, Walker AW, Mansfield J, Parkes M, et al. The Impact of NOD2 Variants on Fecal Microbiota in Crohn's Disease and Controls Without Gastrointestinal Disease. Inflamm Bowel Dis 2018;24:583–92. Available from: https://dx.doi.org/10.1093/ibd/izx061.

ORIGINAL RESEARCH ARTICLES–BASIC SCIENCE

OPEN

The Impact of NOD2 Variants on Fecal Microbiota in Crohn's Disease and Controls Without Gastrointestinal Disease

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Background/Aims: Current models of Crohn's disease (CD) describe an inappropriate immune response to gut microbiota in genetically susceptible individuals. *NOD2* variants are strongly associated with development of CD, and *NOD2* is part of the innate immune response to bacteria. This study aimed to identify differences in fecal microbiota in CD patients and non-IBD controls stratified by *NOD2* genotype.

Methods: Patients with CD and non-IBD controls of known *NOD2* genotype were identified from patients in previous UK IBD genetics studies and the Cambridge bioresource (genotyped/phenotyped volunteers). Individuals with known CD-associated *NOD2* mutations were matched to those with wild-type genotype. We obtained fecal samples from patients in clinical remission with low fecal calprotectin (<250 µg/g) and controls without gastrointestinal disease. After extracting DNA, the V1-2 region of 16S rRNA genes were polymerase chain reaction (PCR)-amplified and sequenced. Analysis was undertaken using the mothur package. Volatile organic compounds (VOC) were also measured.

Results: Ninety-one individuals were in the primary analysis (37 CD, 30 bioresource controls, and 24 household controls). Comparing CD with nonIBD controls, there were reductions in bacterial diversity, *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* and an increase in *Enterobacteriaceae*. No significant differences could be identified in microbiota by *NOD2* genotype, but fecal butanoic acid was higher in Crohn's patients carrying *NOD2* mutations.

Conclusions: In this well-controlled study of *NOD2* genotype and fecal microbiota, we identified no significant genotype-microbiota associations. This suggests that the changes associated with *NOD2* genotype might only be seen at the mucosal level, or that environmental factors and prior inflammation are the predominant determinant of the observed dysbiosis in gut microbiota.

Key Words: Crohn's disease, NOD2, microbiota, genotype

INTRODUCTION

The precise etiology of Crohn's disease (CD) remains unknown. However, the key pathogenic process involves an inappropriate immune response that results in bowel inflammation and damage. The targets of this response are thought to be antigens derived from constituents of the microbiota, a view supported by the benefits of altering the microbiota[1] or physically diverting the fecal stream.[2] Further, 16S rRNA gene sequencing has shown that the microbiota in inflammatory bowel disease (IBD) is abnormal and characterized by reduced diversity with fewer *Firmicutes* species present.[3] The direction of causality between IBD and alterations in microbiota remains incompletely understood, as does the question of whether overall dysbiosis or specific taxa are most important. Recent research also has emphasized the functional aspect of the gut microbiota through measurement of microbial

Received for publications May 4, 2017; Editorial Decision October 2, 2017.

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Conflicts of Interest: None of the authors have any relevant conflicts of interest to report.

§Denotes Joint senior authorship

Supported by: Funding was supported by CORE, the Digestive Diseases Foundation and the Wellcome Trust [grant number 097943 to NAK, 093885 to CAL and 098051 to Alan W Walker and Julian Parkhill . Dr. Walkerreceives core funding support from the Scottish Government Rural and Environmental Science and Analysis Service (RESAS). We also acknowledge the NIHR Biomedical Research Centre awards to Addenbrooke's Hospital/University of Cambridge School of Clinical Medicine and acknowledge the NIHR Newcastle Biomedical Research Centre.

Earlier versions of this work have been presented at British Society of Gastroenterology 2014 (Manchester, UK), BSG 2015 (London, UK), and Digestive Diseases Week 2015 (Washington DC, USA).

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doi: 10.1093/ibd/izx061
Published online 16 February 2018

metabolites such as the volatile organic compounds (VOC) present in feces.[4]

The last few years has seen rapid advances in the genetics of IBD as a result of large cohort genome-wide association studies (GWAS) of cases and controls. Over 200 IBD susceptibility loci have now been reported.[5–7] For some loci, the disease gene and associated point mutations are known (eg, *NOD2* and *ATG16L1*). *NOD2* has the largest effect, and a large recent subphenotype-genotype analysis has confirmed that *NOD2* is strongly associated in particular with ileal CD.[8] Viewed alongside other functionally interrelated genes that have been associated with CD (eg, *ATG16L1*, *IRGM*, and *XBP1*), an impaired capability of the host to regulate microbial constituents consistently emerges as a major common theme.

NOD2 is a cytosolic pattern recognition receptor (PRR) that is a key player in immunity to intracellular bacteria and inflammatory responses. NOD2 recognizes muramyldipeptide (MDP), a ubiquitous component of bacterial cells walls, and its stimulation leads to induction of autophagy in human cells.[9] Variants of *NOD2* associated with CD are mutated in the ligand recognition domain and fail to induce autophagy on MDP triggering, which results in aberrant bacterial handling and antigen presentation in these cells.[10–12] NOD2 possesses other antibacterial effects, including the ability to prime human dendritic cells (DCs) to promote T-helper 17 (Th17) responses (via *NOD2*-induced-expression of IL-23 and IL-1)[13] ,and the ability to induce antimicrobial peptide defensins in the intestine.[14] If expression of CD-variant *NOD2* leads to dysregulated bacterial destruction within the cells in which it is expressed, bacteria may persist abnormally in the mucosa and activate tissue inflammation in these sites.

However, approximately 11%–14% of white Europeans are heterozygous and 0.4%–0.9% homozygous or compound-heterozygous for CD-risk-variant *NOD2* but remain healthy, which reinforces the role for coexistent genetic or environmental factors in initiation of CD.[15,16] The association of defective antibacterial mechanisms with CD-associated polymorphisms in *NOD2* suggest that the presence of these variants may influence the nature of the microbiota over time. This in turn might either lead to a critical dysbiotic state being reached, or the presence of specific microbes emerging to initiate the cycle of inflammation observed in disease. For example, altered release of antibacterial peptides from variant-*NOD2*-expressing Paneth cells, defective Th17 responses, or defective autophagic bacterial processing in the gut mucosa could change gut bacterial burden or species diversity.

Little is known of the nature of the microbiota in the presence of *NOD2* mutations. Human studies to date have been limited in scope due to small numbers of individuals homozygous for *NOD2* mutations without accurate matching of controls. Frank and colleagues revisited the dataset from their index 2007 study on the microbiota in IBD, stratifying patients retrospectively for *NOD2* and *ATG16L1* genotype. Due to its retrospective design, this study is severely constrained by limited power; despite this, they observed clear shifts in microbial composition as a result of genotype.[17] The aim of the current study was to prospectively define the role of *NOD2* genotype in influencing the nature of the host microbiota in health and in CD.

METHODS

Individuals with CD of known *NOD2* genotype were identified from the UK IBD genetics consortium (Fig. 1). Patients were selected if they carried 2 copies of the CD-associated *NOD2* variants [ie, homozygotes or compound heterozygotes for R702W (rs2066844), G908R (rs2066845), or L1007fs (rs2066847)][18,19] as measured using genotyping arrays for the original genetics studies in which they had been involved (Affymetrix GeneChip 500[20] and ImmunoChip[6]). Patients were recruited if they were deemed by their treating physician to be in clinical remission. Each *NOD2*-mutant patient was matched to a homozygous *NOD2*-wild-type patient. Exclusion criteria for CD patients included antibiotics within the months before recruitment, active CD (by physician global assessment), and presence of an ileostomy. For all CD patients, a household control was approached (usually an unrelated spouse). Healthy volunteers stratified by the same *NOD2* variants were recruited from the Cambridge BioResource.[21] The Cambridge BioResource is a panel of around 16,000 volunteers, both with and without health conditions, who have previously submitted DNA for genotyping. Participants can be approached for studies on the basis of genotype and phenotypic characteristics. Volunteers from the BioResource had no known gastrointestinal diagnosis and had not taken antibiotics in the preceeding 3 months. All study participants had fecal calprotectin (FC) measured by a standard ELISA (Calpro AS, Norway). CD patients with FC >250 μg/g and controls (household or BioResource) with FC >50 μg/g were excluded from further analysis.

Clinical data including medical and surgical history, smoking status, medication history, antibiotic use, probiotic use, weight, height, Montreal disease location, and behavior[22] were collected using a patient questionnaire, interrogation of the medical record, and use of previous phenotype information recorded on the IBD cohorts as part of a rephenotyping exercise.[8] Probiotic and medication use were documented at the time of sampling. Participants with missing data were excluded from the analysis of that specific datapoint.

Fecal samples were collected from each study participant using the Fisher Fecal Commode Collection Kit. The collection container was held in the toilet bowl using the supplied trivet, and whole fecal samples collected without contamination by urine. Samples were kept cold using phase-change refrigerant gel packs and processed within 24 hours of collection. This short period of storage is not expected to significantly influence molecular estimation of microbial community composition,[23] nor the VOC profile (unpublished data). Each sample was thoroughly mixed and aliquots were transferred

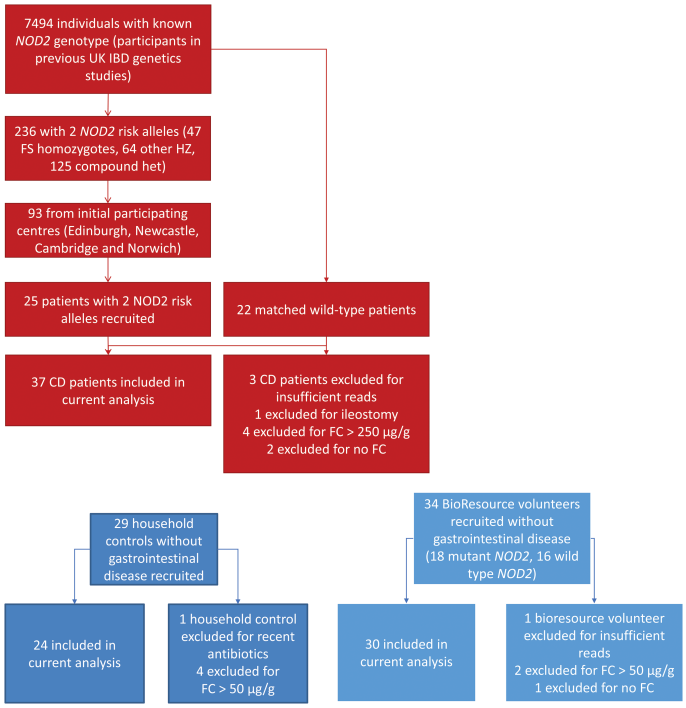

FIGURE 1. Flow diagram of recruitment.

into lysing matrix E tubes (MP Biomedicals, Santa Ana, CA, USA) for subsequent DNA extraction, head space vials (Supelco, Bellefonte, PA, USA) for (VOC) analysis, and universal containers for fecal calprotectin analysis. Samples were stored at −80 °C before shipping to a central processing laboratory in Aberdeen, UK, where DNA was extracted within 1 month of collection.

Household controls had not been previously genotyped. Saliva samples were acquired using Oragene kits (DNA Genotek, Ottawa, Canada). DNA was extracted following the manufacturer's protocol and was genotyped for the 3 CD-associated *NOD2* variants listed above using TaqMan assays (Applied Biosystems, Carlsbad, CA, USA). Where a genotype was not determined, the allelic discrimination plots were examined manually to ensure homozygotes for the minor allele had not been missed. For the purposes of analysis of the household controls, missing genotypes were inferred to be wild-type genotypes.

Ethical Considerations:

Ethical approval was granted by the North of Scotland Research Ethics Committee (reference 12/NS/0050). All participants provided written consent.

DNA Extraction

For each fecal sample, an approximately 400 mg aliquot was placed in a lysing matrix E tube and 978 µl of sodium phosphate buffer and 122 µl MT buffer were added to each tube and vortex mixed. This then was processed using the FastDNA SPIN kit for Soil following the manufacturer's instructions (MP Biomedicals) as described previously.[24]

PCR Amplification and Sequencing

The V1-V2 region of the 16S rRNA gene was amplified using 27F and 338R primers.[25] The primers were designed with the Illumina adapter sequences already included and with 1 of 200 barcode sequences included in the 338R reverse primer, thus avoiding the need for a separate step to add the adapter sequences and barcode. Twenty cycles of polymerase chain reaction (PCR) amplification were performed using the Q5 polymerase kit following the manufacturer's instructions (New England Bio, Ipswich, MA, USA). Postamplification, samples were quantified using a Qubit fluorometer (Thermo Fisher, Waltham, MA, USA) and then pooled to obtain equimolar concentrations.

Sequencing was performed using an Illumina MiSeq sequencer using Illumina V2 chemistry and paired-end 2 × 250 base pair reads. Initial sequence data processing was performed in the Illumina MiSeq Reporter to demultiplex samples and strip adapters and primers and sequence data were exported in the FASTQ format.

Bioinformatics Analysis

The 16S rRNA gene sequence data were further processed using mothur[26] following the MiSeq SOP.[27]

Alignment and classification were done against the SILVA v119 reference set.[28] Community structures were compared using trees generated using Jaccard and Yue Clayton distance metrics after subsampling to 3943 reads per sample. The trees were then plotted graphically using the Interactive Tree of Life.[29,30] Trees were compared using the parsimony command within mothur. Subsequent statistical analysis was done in R 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Microbial diversity was assessed using inverse Simpson and compared using a Mann-Whitney *U* test.[31] Comparisons at the family, genus, and operational taxonomic unit (OTU) level were done using Mann-Whitney *U* tests for binary comparisons and corrected using Holm's method.[32] The 12 most abundant families were selected for plotting graphically.

Volatile Organic Compound Analysis

VOC data were generated using previously described methodology.[33] Briefly, gas chromatography-mass spectroscopy (GCMS) was used to quantify the metabolites in the headspace gas taken from vials containing an aliquot of participants' feces. The raw GCMS data were processed using AMDIS (National Institute of Standards and Technology, Gaithersburg, MD, USA). Compounds detected in fewer than 20% of the study population were filtered out. The resultant ion intensity data were log transformed and the limma package used to facilitate running multiple linear models including disease status and *NOD2* genotype as covariates.[34] *P* values were corrected using Holm's method.

RESULTS

Out of the 110 individuals recruited, 91 were used in the primary analysis (Table 1). Reasons for exclusion are shown in Supplementary Table 1. There were 37 CD patients (57% *NOD2* mutant), 30 bioresource volunteers (58% *NOD2* mutant), and 24 household controls. All were of white European ethnicity. There were no differences in phenotype within the CD patients by *NOD2* status (Table 1). Five of 21 genotyped household controls with genotype information had single CD-associated-*NOD2*-associated mutations. Three of these had a first degree relative with CD.

The total number of raw reads was 3,410,868, with a median number of reads per sample of 34,302. After quality control and removal of samples with very low read numbers, the remaining samples had a minimum of 3943 reads and median of 20,338. The sequence data are available from the European Nucleotide Archive under Study Accession Number PRJEB21593.

There was a significant reduction in diversity (as assessed by calculating the inverse Simpson index) between CD cases and both Bioresource and household controls ($P < 0.001$ and 0.003, respectively, Fig. 2). No difference was observed in diversity by *NOD2* genotype either within the CD cases or the Bioresource controls ($P = 0.32$ and 0.65). Hierarchical clustering using the Jaccard metric demonstrated clustering by CD versus controls in either cohort ($P < 0.001$), but not by *NOD2* genotype ($P = 0.16$ within cases (Fig. 3)). The CD cases also clustered with each other rather than their household controls; indeed the Jaccard distance between cases and their household control was no different from the distance between cases and unmatched household controls ($P = 0.81$, Mann-Whitney *U* test).

At a family level, there were significant decreases in *Ruminococcaceae*, *Rikenellaceae*, and *Christensenellaceae* (*P* all <0.001 uncorrected and <0.01 corrected), and an increase in *Enterobacteriaceae* ($P < 0.001$ corrected) in samples from CD patients vs controls (Fig. 4A). There were no differences in relative abundance of any bacterial families when stratified by NOD2 status, either within the CD patients or Bioresource controls (Fig. 4B). There also were no differences by genotype at the genus or OTU level in each case, comparisons were made using a Mann-Whitney *U* test with correction for multiple testing using Holm's method, and no corrected *P* value was less than 0.05.

Volatile Organic Compound Analysis

For the VOC analysis, there were 314 compounds identified in at least 1 sample, and 198 of those were present in at least 5 CD patients and 5 Bioresource controls.

Linear models were constructed for $\log_2$-transformed data of each compound, with the presence of CD and *NOD2* genotype as the independent variables. These analyses revealed significant reductions in CD patients versus controls in pentanoic acid [$\log_2$ fold change (logFC) −2.11], piperidinone [logFC −2.43], butanone [logFC −2.19], and acetone [logFC −3.90],Table 2].When looking at the effect of carrying 2 of the previously defined *NOD2* mutations, there was a single significant association after correction for multiple testing using the Holm's method with butanoic acid (logFC 1.25, corrected $P = 0.024$). On further examination, this VOC was noted to be less abundant specifically in patients with CD with wild-type *NOD2* (Fig. 5).

DISCUSSION

This prospective study examines the relationship between *NOD2* genotype and the fecal microbiota in human participants

TABLE 1: Study Demographics

A: Whole Cohort					
		CD Patients (n = 37)	Bioresource Controls (n = 30)	Household Controls (n = 24)	*P*
Sex: Female		23 (62%)	16 (53%)	9 (38%)	0.18
Age/years		53 (44–65)	60 (52–64)	51 (43–61)	0.30
BMI		23.5 (21.7–27.2)	25.4 (23.0–27.5)	25.0 (22.8–28.2)	0.12
Probiotic use		3 (8%)	3 (10%)	0 (0%) (1 not recorded)	0.37
Antibiotics within past 12 months (but >3 months)		17 (47%) (1 not recorded)	4 (13%)	9 (39%) (1 not recorded)	0.01
Smoking	Current	5 (14%)	1 (3%)	0 (0%)	0.03
	Ex	18 (49%)	8 (27%)	12 (52%)	
	Never	14 (38%)	21 (70%)	11 (48%)	
B: CD subcohort (from UK IBD genetics consortium)					
			Wild- type *NOD2* (n = 16)	Mutant *NOD2* (n = 21)	*P*
Female Sex			10 (62%)	14 (64%)	1.00
Age/years			56 (46–66)	52 (41–64)	0.36
Smoking	Current		2 (12%)	3 (14%)	0.81
	Ex		9 (56%)	9 (43%)	
	Never		5 (31%)	9 (43%)	
Montreal location	L1		9 (60%)	8 (44%)	0.35
	L2		0 (0%)	3 (17%)	
	L3		7 (40%)	7 (39%)	
	Unknown		1	3	
Montreal behaviour	B1		3 (20%)	4 (22%)	0.73
	B2		9 (60%)	8 (44%)	
	B3		3 (20%)	6 (33%)	
	Unknown		1	3	
History of surgical resection for IBD			15 (94%)	19 (90%)	1.00
Current 5-aminosalicylate or sulphasalazine			3 (19%)	7 (33%)	0.46
Current immunomodulator			8 (50%)	5 (24%)	0.17

Data are presented as medians and interquartile range, or numbers and percentages as appropriate. *P* values are Kruskal Wallis for continuous variables and Fisher's exact test for categorical variables.

stratified by *NOD2* genotype. It further confirms previously identified shifts in gut microbiota in CD patients when compared to non-IBD controls, notably a reduction in obligate anaerobic lineages in tandem with an increase in the facultatively anaerobic *Enterobacteriaceae* family. These changes have previously been described in both inflamed and uninflamed tissue and in both fecal and mucosal samples.[35–38] However, no significant differences in fecal microbiota were seen when analysed by *NOD2*-status, at any of the taxonomic levels assessed. The present study also includes VOC data and demonstrates the value of having a means to assess the functional aspects of the gut microbiota, and we were able to demonstrate higher butanoic acid concentrations in CD patients with *NOD2* mutations than those without.

Earlier animal studies have shown an association between *NOD2* genotype and gut microbiota. Both Rehman et al and Mondot et al showed reductions in diversity and changes in specific taxa when comparing wild-type and *NOD2* knockout mice.[39,40] However, more recently, Shanahan et al conducted experiments where the knockout and wild-type mice were cohoused and failed to demonstrate a *NOD2* genotype-specific effect on gut microbiota. They concluded that the cage environment was more important than genotype. Carmody et al went further and looked at the relative impact of genotype and diet on the gut microbiota in mice; they demonstrated dominant effects of diet, regardless of the underlying host genetics.[41] Nonetheless, Nabhani et al found *NOD2*-related differences in mucosal microbiota even when

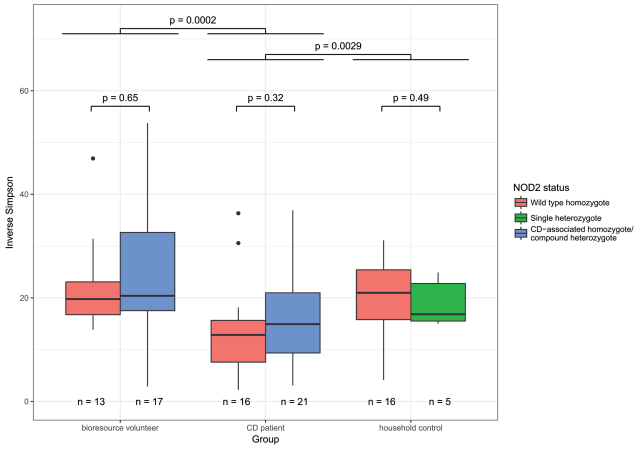

FIGURE 2. Inverse Simpson index of microbial diversity by *NOD2* status and case type.

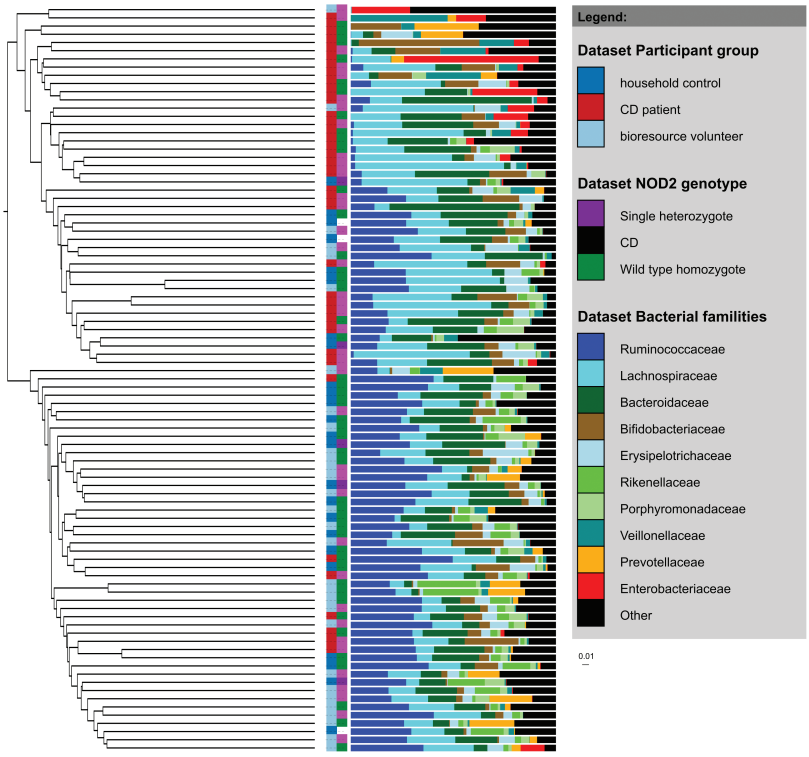

FIGURE 3. Hierarchical clustering by Jaccard distance metric of the 16S rRNA gene data showing differences by study group and *NOD2* status. The panel on the right shows the relative proportions of the 10 most prevalent bacterial families and the cumulative relative proportion of all other bacteria (shown in black).

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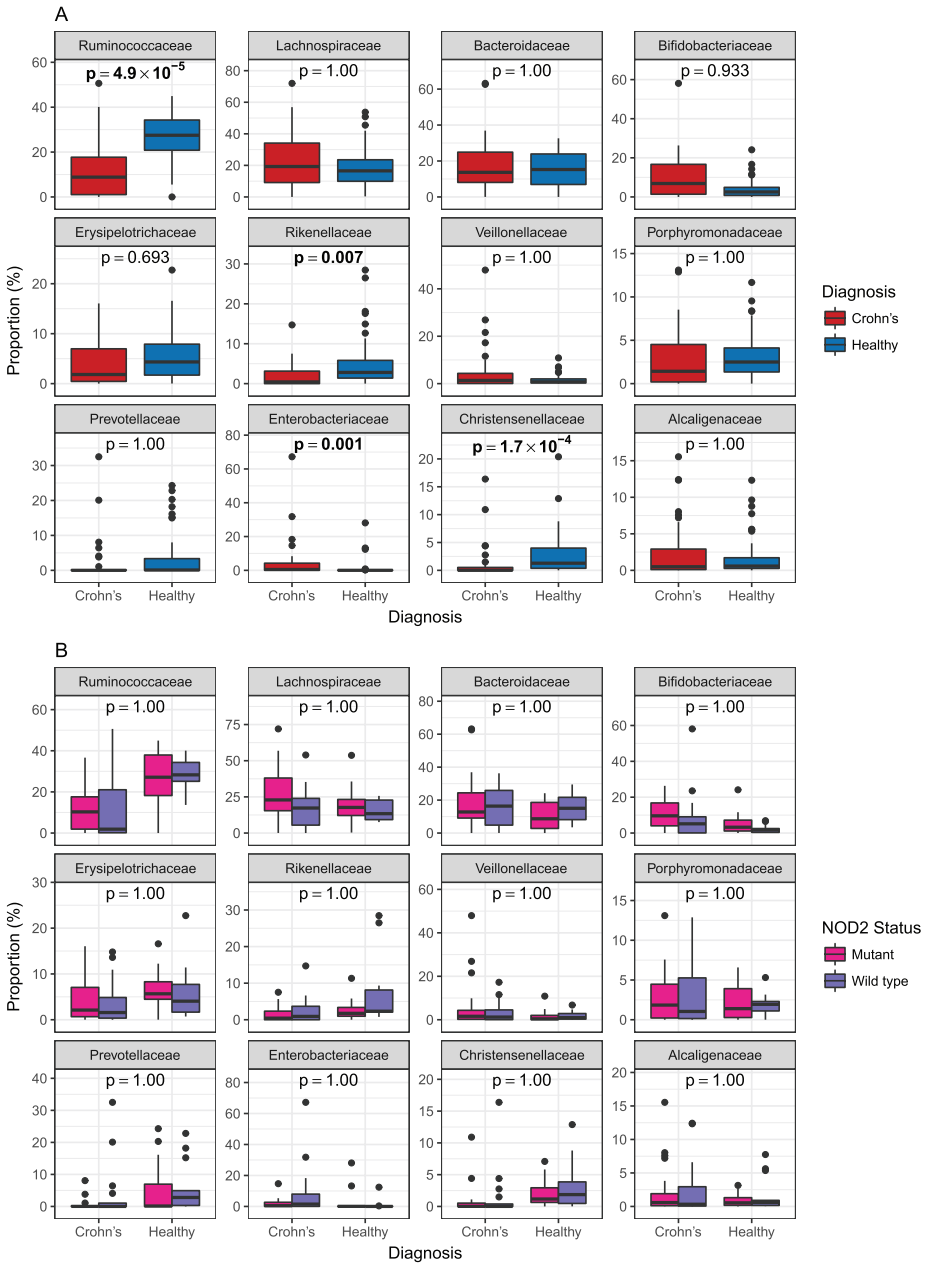

FIGURE 4. A, Relative abundance of the 12 most prevalent bacterial families in both CD patients and non-IBD controls. *P* values are corrected for multiple testing using Holm's method across all 59 families seen in the sequencing data. Corrected *P* values < 0.05 are highlighted in bold. B, Relative abundance of the 12 most prevalent bacterial families where samples have been grouped by diagnosis and by *NOD2* genotype. *P* values are corrected for multiple testing using Holm's method across all 59 families seen in the sequencing data. Mutant *NOD2* is defined here as the presence of 2 CD associated mutations (rs2066844, rs2066845, rs2066847); wild-type *NOD2* is defined as the absence of any of these mutations.

TABLE 2: Top Volatile Organic Compounds by Presence of CD [a]

Compound	Log_2 fold change	*P*	Holm-corrected *P*
Pentanoic acid	-3.29	2.2×10^{-8}	2.5×10^{-6}
2-Piperidinone	2.10	1.7×10^{-7}	2.0×10^{-5}
2-Butanone	-2.57	3.9×10^{-7}	4.4×10^{-5}
Dimethyl sulfide	-2.47	1.3×10^{-6}	1.5×10^{-4}
Acetone	-2.25	1.6×10^{-6}	1.8×10^{-4}
1H-Indole, 3-methyl-	-4.03	2.3×10^{-6}	2.5×10^{-4}
Butanoic acid, 3-methyl-, ethyl ester	2.13	3.5×10^{-6}	3.8×10^{-4}
Furan, 2-methyl-	-1.57	1.1×10^{-5}	0.001
2-Hexanone, 5-methyl-	-1.50	6.1×10^{-5}	0.006
Butanoic acid, 2-methyl-, ethyl ester	1.90	1.3×10^{-4}	0.013

[a]Derived from linear model of all CD and non-IBD patients with CD and *NOD2* genotype as covariates.

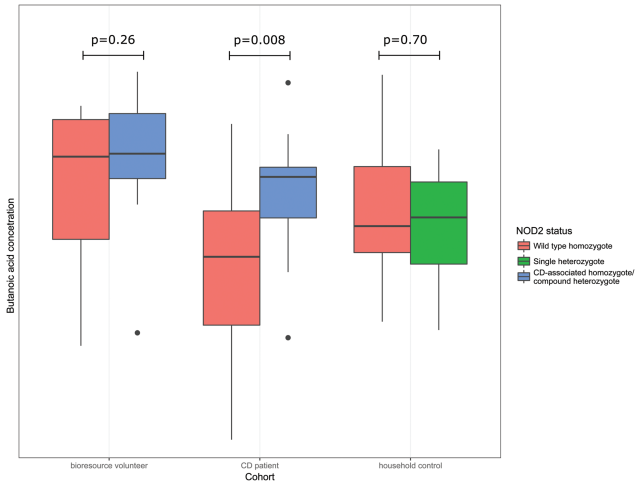

FIGURE 5. Concentration of butanoic acid stratified by cohort and by *NOD2* status. *P* values shown are uncorrected and are for Mann-Whitney *U* tests by *NOD2* status within each cohort. Mutant *NOD2* is defined here as the presence of 2 CD associated mutations (rs2066844, rs2066845, rs2066847); wild-type *NOD2* is defined as the absence of any of these mutations.

NOD2-knockout and wild-type mouse embryos were mixed and transferred to wild-type surrogates and were subsequently cohoused.[42]

In humans, others have previously reported an effect of *NOD2* on intestinal microbiota. Knights et al reported results from cohorts comprising a total of 474 individuals with IBD, though not stratified by *NOD2* status.[43] They identified an association between 6 causal *NOD2* variants and increased *Enterobacteriaceae* measured in intestinal biopsies. Of note, they were able to identify similar patterns in ulcerative colitis patients with *NOD2* mutations, suggesting that the observed effect is not just one of disease phenotype. However, in a network of associations between bacterial taxa, host, and environmental factors, the effect of *NOD2* genotype was only modest compared to antibiotic usage, immunosuppressants, biopsy location, and cohort of origin. Li et al reported differences in intestinal biopsy microbial profile related to *NOD2* genotype alongside disease phenotype, with an increase in the *C. coccoides-E. rectales* group in patients with ileal CDcarrying a risk *NOD2* allele.[44] More recently, Imhann et al reported an interaction between an IBD genetics risk score that included *NOD2* variants and the

fecal microbiota, although the impact of *NOD2* on its own was not described.[45]

Strengths of this present study include the use of patients and nonIBD controls of known *NOD2* genotype, with close matching of the phenotypic characteristics across genotypes. Establishing the causal relationship between the gut microbiota and IBD remains challenging; intestinal inflammation is well established as a cause of dysbiosis.[46] The study excluded participants with either clinical or biomarker evidence of active disease, reducing the possibility of confounding by disease activity. Although the use of patients in remission will have removed one source of variability, it is also possible that the effects of *NOD2* are manifest during active disease. With regards to limitations, this study explores only the changes in gut microbiota in the fecal contents, which are unlikely to fully reflect changes at the mucosal level, particularly in the terminal ileum where one might expect *NOD2* to exert its strongest effect. This reflects the difficulty in accessing colonoscopic biopsy samples in a cohort of non-IBD controls and patients in remission. Although only a single VOC was significantly different by *NOD2* status, this suggests a possibile difference in metabolically active bacteria not well-represented in fecal samples. The patient cohort also had well-established disease, with a history of surgical resection in most participants. This may reflect a higher risk of surgical resection in patients with *NOD2* mutations, noting that the wild-type controls were matched using this phenotype among others. Shotgun metagenomic analysis might have facilitated detection of differences at the species or gene level between cohorts that could be missed with 16S rRNA taxonomic analysis.

CONCLUSION

This study confirms associations between altered fecal microbiota and Crohn's disease, but failed to identify any differences in microbiota between individuals stratified by NOD2 genotype. Future studies should explore the relationship between *NOD2* genotype and ileal-associated bacteria, ideally using either cohorts again stratified by genotype or very large cohorts to generate adequate numbers of individuals carrying 2 disease-associated mutations. Large cohort studies also offer the opportunity to perform more extensive genotype-microbiota-phenotype analyses, which should lead to a better understanding of these complex interactions.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

ACKNOWLEDGEMENTST

Nicholas A Kennedy , Georgina L Hold , Charlie W Lees, Alan W Walker , Julian Parkhill and Chris Probert designed the study. Nicholas A Kennedy and Charlie W Leescoordinated the study. Nicholas A Kennedy , Christopher A Lamb , John Mansfield, Miles Parkes, Rachel Simpkins, Mark Tremelling, Sarah Nutland, and Charlie W Lees recruited patients from the study sites. Susan H Berry, Georgina L Hold, and Nicholas A Kennedy prepared the collected samples for sequencing and measurement of calprotectin. Chris Probert generated and analysed volatile organic compound data. Alan W Walker and Julian Parkhill generated 16S rRNA sequencing data. Nicholas A Kennedy analysed the clinical and scientific data and wrote the initial draft of the paper with input from Georgina L Hold and Charlie W Lees . All authors reviewed and contributed to subsequent drafts of the paper and approved the final manuscript.

REFERENCES

1. Kajiura T, Takeda T, Sakata S, et al. Change of intestinal microbiota with elemental diet and its impact on therapeutic effects in a murine model of chronic colitis. *Dig Dis Sci*. 2009;54:1892–1900.
2. Winslet MC, Andrews H, Allan RN, et al. Fecal diversion in the management of Crohn's disease of the colon. *Dis Colon Rectum*. 1993;36:757–762.
3. Frank DN, St Amand AL, Feldman RA, et al. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A*. 2007;104:13780–13785.
4. Ahmed I, Greenwood R, Costello B, et al. Investigation of faecal volatile organic metabolites as novel diagnostic biomarkers in inflammatory bowel disease. *Aliment Pharmacol Ther*. 2016;43:596–611.
5. Liu JZ, van Sommeren S, Huang H, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. *Nat Genet*. 2015;47:979–986.
6. Jostins L, Ripke S, Weersma RK, et al.; International IBD Genetics Consortium (IIBDGC). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012;491:119–124.
7. de Lange KM, Moutsianas L, Lee JC, et al. Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. *Nat Genet*. 2017;49:256–261.
8. Cleynen I, Boucher G, Jostins L, et al.; International Inflammatory Bowel Disease Genetics Consortium. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: a genetic association study. *Lancet*. 2016;387:156–167.
9. Philpott DJ, Sorbara MT, Robertson SJ, et al. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol*. 2014;14:9–23.
10. Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med*. 2010;16:90–97.
11. Travassos LH, Carneiro LA, Ramjeet M, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol*. 2010;11:55–62.
12. Homer CR, Richmond AL, Rebert NA, et al. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology*. 2010;139:1630–1641, 1641.e1.
13. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, et al. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote Interleukin-17 production in human memory T cells. *Immunity*. 2007;27:660–669.
14. Kobayashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*. 2005;307:731–734.
15. MacArthur D, Palotie A, Metspalu A, et al. gnomAD browser Gene: NOD2. http://gnomad.broadinstitute.org/gene/ENSG00000167207 (29 June 2017, date last accessed).
16. Yazdanyar S, Kamstrup PR, Tybjaerg-Hansen A, et al. Penetrance of NOD2/CARD15 genetic variants in the general population. *CMAJ*. 2010;182:661–665.
17. Frank DN, Robertson CE, Hamm CM, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. *Inflamm Bowel Dis*. 2011;17:179–184.
18. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature*. 2001;411:603–606.
19. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*. 2001;411:599–603.
20. Burton PR, Clayton DG, Cardon LR, et al. Genome-wide association study of 14,000 cases of seven common diseases and 3000 shared controls. *Nature*. 2007;447:661–678.

21. Cambridge Bioresource. Introducing the Cambridge BioResource—The Cambridge NIHR BioResource. 2017. http://www.cambridgebioresource.group.cam.ac.uk/.
22. Silverberg MS, Satsangi J, Ahmad T, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a working party of the 2005 montreal world congress of gastroenterology. *Can J Gastroenterol*. 2005;19(Suppl A):5A–36A.
23. Cardona S, Eck A, Cassellas M, et al. Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiol*. 2012;12:158.
24. Kennedy NA, Walker AW, Berry SH, et al. The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PLoS One*. 2014;9:e88982.
25. Bernardo D, Durant L, Mann ER, et al. Chemokine (C-C motif) receptor 2 mediates dendritic cell recruitment to the human colon but is not responsible for differences observed in dendritic cell subsets, phenotype, and function between the proximal and distal colon. *Cell Mol Gastroenterol Hepatol*. 2016;2:22–39.e5.
26. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*. 2009;75:7537–7541.
27. Schloss PD. MiSeq SOP—mothur. 2015. http://www.mothur.org/wiki/MiSeq_SOP (20 March 2015, date last accessed).
28. Pruesse E, Quast C, Knittel K, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res*. 2007;35:7188–7196.
29. Letunic I, Bork P. Interactive tree of life (itol): an online tool for phylogenetic tree display and annotation. *Bioinformatics*. 2007;23:127–128.
30. Letunic I, Bork P. Interactive tree of life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res*. 2011;39:W475–W478.
31. Simpson EH. Measurement of diversity. *Nature*. 1949;163:688.
32. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Stat*. 1979;6:65–70.
33. Aggio RB, Mayor A, Reade S, et al. Identifying and quantifying metabolites by scoring peaks of GC-MS data. *BMC Bioinformatics*. 2014;15:374.
34. Smyth GK. Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, et al., eds. *Bioinformatics and Computational Biology Solutions Using {R} and Bioconductor*. New York: Springer; 2005:397–420.
35. Hold GL, Smith M, Grange C, et al. Role of the gut microbiota in inflammatory bowel disease pathogenesis: what have we learnt in the past 10 years? *World J Gastroenterol*. 2014;20:1192–1210.
36. Walker AW, Sanderson JD, Churcher C, et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. *BMC Microbiol*. 2011;11:7.
37. Gevers D, Kugathasan S, Denson LA, et al. The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe*. 2014;15:382–392.
38. Papa E, Docktor M, Smillie C, et al. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. *Plos One*. 2012;7:e39242.
39. Mondot S, Barreau F, Al Nabhani Z, et al. Altered gut microbiota composition in immune-impaired Nod2(-/-) mice. *Gut*. 2012;61:634–635.
40. Rehman A, Sina C, Gavrilova O, et al. Nod2 is essential for temporal development of intestinal microbial communities. *Gut*. 2011;60:1354–1362.
41. Carmody RN, Gerber GK, Luevano JM Jr, et al. Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe*. 2015;17:72–84.
42. Al Nabhani Z, Lepage P, Mauny P, et al. Nod2 deficiency leads to a specific and transmissible Mucosa-associated microbial dysbiosis which is independent of the mucosal barrier defect. *J Crohn's Colitis*. 2016;10:1428–1436.
43. Knights D, Silverberg MS, Weersma RK, et al. Complex host genetics influence the microbiome in inflammatory bowel disease. *Genome Med*. 2014;6:107.
44. Li E, Hamm CM, Gulati AS, et al. Inflammatory bowel diseases phenotype, C. Difficile and NOD2 genotype are associated with shifts in human ileum associated microbial composition. *PLoS One*. 2012;7:e26284.
45. Imhann F, Vich Vila A, Bonder MJ, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut*. 2016;doi: 10.1136/gutjnl-2016-312135.
46. Buttó LF, Haller D. Dysbiosis in intestinal inflammation: cause or consequence. *Int J Med Microbiol*. 2016;306:302–309.

5.3 Discussion of paper

In this study, I was able to confirm some of the associations between Crohn's disease and changes in the faecal microbiota, even in patients in remission. *NOD2* was chosen here for two important reasons. Firstly, it remains one of the genetic association with Crohn's disease that has highest effect size.[13] Secondly, *NOD2* is an intracellular receptor that recognises bacterial peptidoglycan, specifically muramyl dipeptide, and triggers innate immune responses.[14] All of the genetic variants tested in this study are in the leucine-rich repeat area which is highly conserved and is important for ligand binding. They are thought to increase the risk of Crohn's disease through dysregulation of the immune response to gut microbiota. It is therefore reasonable to think that there may have been an association between loss-of-function genetic variants and the relative abundance of luminal bacteria.

I was unable to demonstrate a significant impact of host *NOD2* genotype on the bacterial taxa observed, despite an adequately-powered study. This may indicate that associations described elsewhere in the literature are only manifest during active disease, or that they are confined to the ileum which is less well represented by the bacterial contents of faecal samples.[133] It is also possible that *NOD2* modulates the host response to bacteria but does not influence the composition of the gut microbiota. Nonetheless, this study provides further insights into understanding the relationship between host genetics and the gut microbiota. Interestingly, a subsequent study by Aschard *et al.* has successfully demonstrated an association of changes in Roseburia with *NOD2* genotype in a cohort of 182 patients with IBD. However, about half of the included patients had active disease at the type of sampling.

6 Clinical utility and diagnostic accuracy of faecal calprotectin for IBD at first presentation to gastroenterology services in adults aged 16–50 years

6.1 Introduction to paper

Alongside developing a better understanding of disease pathogenesis, I have been keen to explore how we can better use both existing and novel techniques to diagnose Crohn's disease and to predict outcomes. Faecal calprotectin is the main cytosolic protein in neutrophils and has emerged as a useful tool for measuring inflammation in a variety of bodily fluids,[134–136] but particularly in faecal samples.[137] Edinburgh was a relatively early adopter of faecal calprotectin in routine clinical practice and has been analysing samples since 2005. In this project, I analysed data from almost 900 patients who had had calprotectin measured as part of their presentation to secondary gastroenterology services. This differed from most of the published literature prior to the study, since prior studies were mostly either small in size, or used patients with established diagnoses of IBD and irritable bowel syndrome (IBS).

6.2 Contributions

This study was originally conceived by Charlie Lees. I collected data working alongside Annalie Clark, Andrew Walkden, Jeff Chang, Federica Fascí-Spurio and Martina Muscat. The calprotectins were measured in the Western General Hospital Biochemistry Department. I developed the analytical strategy and performed the data analysis. I wrote the paper, under the supervision of Charlie Lees, and with support from Annalie Clark and Andrew Walkden.

Published as: Chapter 4.Kennedy NA, Clark A, Walkden A, Chang JCW, Fascí-Spurio F, Muscat M, et al. Clinical utility and diagnostic accuracy of faecal calprotectin for IBD at first presentation to gastroenterology services in adults aged 16-50 years. J Crohns Colitis 2015;9:41–9. Available from: https://dx.doi.org/10.1016/j.crohns.2014.07.005.

Journal of Crohn's and Colitis, 2015, 41–49
doi:10.1016/j.crohns.2014.07.005
Original Article

Original Article

Clinical Utility And Diagnostic Accuracy of Faecal Calprotectin For IBD At First Presentation To Gastroenterology Services In Adults Aged 16–50 Years

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Received February 3 2014; revised July 2 2014; accepted July 11 2014.

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Abstract

Background: Distinguishing inflammatory bowel disease (IBD) from functional gastrointestinal (GI) disease remains an important issue for gastroenterologists and primary care physicians, and may be difficult on the basis of symptoms alone. Faecal calprotectin (FC) is a surrogate marker for intestinal inflammation but not cancer.
Aim: This large retrospective study aimed to determine the most effective use of FC in patients aged 16–50 presenting with GI symptoms.
Methods: FC results were obtained for patients presenting to the GI clinics in Edinburgh between 2005 and 2009 from the Edinburgh Faecal Calprotectin Registry containing FCs from >16,000 patients. Case notes were interrogated to identify demographics, subsequent investigations and diagnoses.
Results: 895 patients were included in the main analysis, 65% female and with a median age of 33 years. 10.2% were diagnosed with IBD, 7.3% with another GI condition associated with an abnormal GI tract and 63.2% had functional GI disease. Median FC in these three groups were 1251, 50 and 20 μg/g ($p < 0.0001$). On ROC analysis, the AUC for FC as a predictor of IBD vs. functional disease was 0.97. Using a threshold of ≥ 50 μg/g for IBD vs. functional disease yielded a sensitivity of 0.97, specificity of 0.74, positive predictive value of 0.37 and negative predictive value of 0.99. Combined with alarm symptoms, the sensitivity was 1.00.
Conclusions: Implementation of FC in the initial diagnostic workup of young patients with GI symptoms, particularly those without alarm symptoms, is highly accurate in the exclusion of IBD, and can provide reassurance to patients and physicians.

Keywords: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Faecal calprotectin; Diagnostic test; Sensitivity

Abbreviations

AUC	area under the curve
CD	Crohn's disease
CRP	C-reactive protein
ESR	erythrocyte sedimentation rate
FC	faecal calprotectin
IBD	inflammatory bowel disease
IBDU	inflammatory bowel disease unclassified
NHS	National Health Service
NICE	National Institute of Clinical Excellence
NPV	negative predictive value
PPV	positive predictive value
ROC	receiver operating curve
UC	ulcerative colitis

1. Introduction

The relatively non-specific clinical manifestations of gastrointestinal disease can make it difficult for clinicians to distinguish between functional and organic intestinal disease, especially in patients presenting without rectal bleeding or systemic upset.[1,2] The gold standard for identifying bowel inflammation, colonoscopy and histology, is an expensive and invasive procedure. Although attitudes to clinical targets have changed, endoscopic services are limited in many countries and a non-invasive tool to select individuals for early referral and investigations would enable the most cost effective use of resources.

Faecal calprotectin (FC), a 36.5 kDa calcium-binding cytosolic protein found in neutrophils, is increasingly being used in clinical practice as a surrogate marker for intestinal inflammation. FC correlates with faecal excretion of white cells and a number of studies have demonstrated that FC is significantly elevated in the stool of patients with active inflammatory bowel disease (IBD) compared to control groups.[3–6] There is a large amount of existing literature relating to FC and its use in differentiating IBD and irritable bowel syndrome (IBS). However, the majority of these studies use data obtained from patients with a pre-existing diagnosis of IBD and IBS. Few studies assess the use of FC in undiagnosed populations; those that do analyze small sample sizes.[3,5–9] FC is described by the British Society for Gastroenterology IBD guidelines as accurate in detecting colonic inflammation, and a NICE review was completed in October 2013.[10,11] The systematic review that has been produced as part of this assessment reported that 'calprotectin testing will lead to considerable savings to the NHS, as well as the avoidance of an unpleasant invasive procedure in people whose symptoms are due to IBS.'[12]

The current recommended upper limit of FC in the faeces of healthy individuals is 50 µg/g. A meta-analysis of adult patients has previously given sensitivity of 95% and a specificity of 91% when using a 50 µg/g cut-off threshold for differentiating IBD from healthy controls.[13] A more recent meta-analysis of prospective studies using patients with suspected IBD found the pooled sensitivity and specificity of FC to be 93% and 96% respectively, although this analysis used studies with variable cut off values ranging from 24 to 150 µg/g.[14] Importantly, FC is a poor test for colorectal cancer with a sensitivity and specificity of 36% and 71% respectively.[13] However FC could potentially be used in clinical practice to identify young adult patients who require further invasive investigation to exclude intestinal inflammation. When used in the correct clinical scenario, with no alarm symptoms present, a negative FC result could be highly suggestive of an absence of organic gastrointestinal disease, thus usually avoiding the need for invasive investigation. Patients over the age of 50 years presenting with lower GI symptoms will require colonoscopy to exclude colorectal cancer.

Since 2005 a reliable FC assay has been available in the biochemistry department at the Western General Hospital, Edinburgh. More than 8000 assays had been performed by 2008. Our clinical practice has evolved to utilise FC values in two main areas. First, FC has been used to monitor disease activity in patients with established IBD; second, to exclude IBD in patients presenting to the out-patient clinic with lower GI upset. As confidence in the utility of FC has grown, we have tended in recent years to avoid invasive endoscopic and radiological investigation in young adult patients with a negative FC (< 50 µg/g) and no alarm symptoms.

This study aimed to determine the most effective use of FC in the diagnosis of GI disease in patients with no prior known GI disease, at the first presentation to GI services. We assessed how FC can be used as a non-invasive tool to aid referral to GI services, and how this improves cost effectiveness of resource allocation through reduction of unnecessary colonoscopies. Comparison was made against other serum markers to determine the optimal initial diagnostic workup of patients aged 16–50 years.

2. Methods

2.1 Patient population

Patient data were analysed from two large teaching hospitals within the same healthcare board (NHS Lothian): Western General Hospital (WGH), Edinburgh, and the Royal Infirmary Edinburgh (RIE). These were identified using the Edinburgh Faecal Calprotectin Register (EFCR), a record kept by the Biochemistry department at WGH. The EFCR contains the name, patient I.D., date of birth, referring hospital/department, and FC concentration for all of the samples analysed.

2.2 Derivation of cohort

The EFCR contains the data of 22,204 FC samples from 16,267 patients (Fig. 1). Patients were identified who had had their first FC between January 2005 and April 2009 to allow sufficient follow-up time to pick up cases of latent IBD. 1544 patients were aged 50 or under and had at least one sample originating from the WGH or RIE from the index period. Where multiple FC samples were listed for the same patient, the initial FC value from the patient's first presentation was included in all analyses. Subjects were excluded from the study if they had a confirmed GI diagnosis at time of sample (n = 247) or if they had already started treatment for presumed IBD (n = 14).

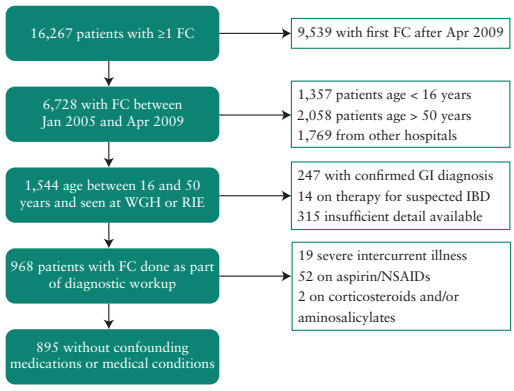


Figure 1. Derivation of the cohort.

For the primary analysis, patients suffering from severe intercurrent illnesses (n = 19) were excluded as were patients receiving NSAID or aspirin therapy (n = 52), aminosalicylates and/or corticosteroids (n = 2), leaving 895 patients in the final cohort.

2.3 FC assay technique

FC was measured in a faecal extract using a standard enzyme-linked immunosorbent assay (ELISA) technique as previously described (Calpro AS, Norway).[15] Faecal extract was added to a microtitre plate pre-coated with polyclonal antibodies to FC. Bound FC was detected using an alkaline phosphatase labelled human antibody to FC and quantified by comparison with a known standard preparation (numerical values given between 20 and 2500 μg/g). This assay was performed in the Department of Clinical Biochemistry at the Western General Hospital, Edinburgh. The reported assay precision for the calprotectin ELISA is a coefficient of variation (CV) of less than 6%. When including the faecal extraction step, the CV for the entire assay has been estimated to be less than 10% (unpublished data; 2014 email from Susan Walker, Department of Clinical Biochemistry, Western General Hospital, Edinburgh).

2.4 Data collection

Data was collected retrospectively by review of electronic patient records and recorded on a standardised data collection form. The electronic patient record system (Trak, Intersystems, Cambridge MA, USA) logs all patient contacts with secondary care (throughout NHS Lothian), including all endoscopic and radiological investigations, clinic appointments and hospital admissions. This data was then cross-referenced with other hospital electronic databases that store clinic letters and laboratory results in order to ensure the maximum retrieval and accuracy of data. Patients were followed up until at least three years after first presentation using Trak, ensuring all re-presentations and subsequent diagnoses were noted.

Parameters recorded were: age, gender, FC level and date of sample, presenting complaints (bloody diarrhoea, watery diarrhoea, rectal bleeding, constipation, abdominal pain, weight loss, flatulence/bloating, vomiting, dyspepsia, fatigue, possible extraintestinal manifestations, other), past medical history, family history (ulcerative colitis (UC), Crohn's disease (CD), IBD unclassified (IBDU), coeliac disease, colon cancer), smoking history (current at time of FC, ex- or never), drug history (including NSAIDs, antibiotics, laxatives, opioids, immunosuppresants, loperamide, aminosalicylates, acetaminophen, aspirin, corticosteroids), investigations performed (stool culture, colonoscopy, flexible sigmoidoscopy, upper GI endoscopy, abdominal ultrasound scan, abdominal X-ray, small bowel MRI, abdominal/pelvic CT, barium enema, barium follow-through, capsule endoscopy and radiolabelled white cell scan) and blood results (full blood count, liver function tests, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), albumin, hematinics (ferritin, vitamin B12 and serum folate), thyroid function tests, glucose, 7 alphahydroxycholestenone and anti-tissue transglutaminase IgA titre). Rectal bleeding, bloody diarrhoea, nocturnal symptoms, weight loss and anaemia were grouped as "alarm symptoms". Where a laboratory test was reported as greater or less than a threshold, for statistical purposes it was assigned to one more or one less than the threshold respectively.

Any investigations performed were recorded as normal, abnormal or incomplete. "Abnormal" endoscopy findings included mucosal abnormalities, such as histologically proven malignancies and inflammation. The normal group includes those where no abnormalities were found as well as non-adenomatous polyps and haemorrhoids.

2.5 Diagnosis

Diagnosis was recorded as had been stated in the clinical notes. The Lennard-Jones criteria were used to diagnose IBD and the Montreal criteria to classify clinical phenotypes.[16,17] The ROME III criteria were used to classify patients diagnosed with IBS.[18] In cases where a diagnosis had not been recorded in the clinical notes, anonymised patient's notes were reviewed independently by two gastroenterologists blinded to the FC level (CWL and IDRA). Organic GI diagnoses were grouped as IBD, 'abnormal gastrointestinal (GI) tract' where a diagnosis would be expected to demonstrate a macroscopically abnormal GI tract and other GI where bidirectional endoscopy and capsule endoscopy would be expected to be normal. Details can be seen in Table S1.

Patients with a definitive organic diagnosis or who had undergone full colonoscopy (n = 467) were censored at the time of initial case note review. Those cases where an organic diagnosis was not made at the time of the FC or where no colonoscopy had been performed (n = 428) were reviewed in the last quarter of 2012 to ensure that no further cases of IBD or other significant GI pathology had been missed. Patients whose symptoms resolved spontaneously, who did not require further investigation and who did not re-present to hospital with GI symptoms were classified as 'symptoms resolved.' Those who were lost to follow-up without a definitive diagnosis were classed as 'lost to follow-up'.

The main comparisons have been made in those with functional disease vs. those with IBD or another condition associated with an abnormal tract, since these are the patients in whom endoscopy would be a potentially useful test.

2.6 Cost analysis

Potential cost savings were calculated using 2012 tariff prices quoted by the Department of Health.[19] One colonoscopy with biopsies in a patient aged 19 years or older was stated to cost £563, while a flexible sigmoidoscopy plus biopsy cost £360. The in-house processing cost of a single FC assay at WGH in 2008 was £24.47.

2.7 Statistical analysis

Statistical analyses of functional vs. organic groups and functional vs. IBD groups were performed. Medians and inter-quartile range are provided. Mann–Whitney U, Kruskal–Wallis, chi-squared and Fisher's exact tests were used to determine statistical significance. Receiver operating characteristic (ROC) curves were used to determine the best cut off point for FC when predicting organic disease and IBD. Comparison of area under the curve (AUC) was performed using the Delong and bootstrap methods. Positive predictive values (PPV) and negative predictive values (NPV) were calculated. Pre-test probabilities were calculated using all individuals regardless of FC concentration. Post-test probabilities were calculated with respect to different thresholds of FC.

A two-tailed p-value of less than 0.05 was considered significant. Confidence intervals for sensitivity and specificity were calculated using the method described by Newcombe with continuity correction.[20] Confidence intervals for likelihood ratios were calculated using the method described by Simel et al.[21] Statistical analyses were performed using R 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1 Demographics

64.9% of patients were female, and the median age (interquartile range) at the time of FC was 33.1 years (25.6–40.7) (Table 1). 566/895 (63.2%) of patients were diagnosed with a functional disorder

(Table 2). 91/895 (10.2%) were diagnosed with IBD, while a further 58 (7.3%) had conditions associated with an abnormal gastrointestinal tract. 63 patients (7.0%) had other miscellaneous gastrointestinal disorders. 78 patients (8.7%) did not have a final diagnosis, of whom 48 had complete symptomatic resolution and have not re-presented in ≥ 3 years, one has had further presentations with abdominal pain without a diagnosis while the remainder were lost to follow-up.

3.2 FC and demographic variables

FC was not significantly associated with age (p = 0.21), sex (p = 0.18) or current smoking (p = 0.80).

3.3 FC and other clinical parameters assessed by final diagnosis

FC was significantly higher in patients diagnosed with IBD (median FC 1251 µg/g, IQR 532–2325 µg/g) than those with other conditions associated with an abnormal gastrointestinal tract (median FC 50 µg/g, IQR 20–145 µg/g) or with a functional diagnosis (median FC 20 µg/g, IQR < 20–50 µg/g) (p ≤ 0.0001 in each case, see Fig. 2).

3.4 FC in patients taking non-steroidal anti-inflammatories (NSAIDs)

Patients taking NSAIDs or aspirin were excluded from the primary analysis. In patients with a functional diagnosis, the FC was significantly higher in those taking NSAIDs or aspirin when compared with those on neither drug (median FC 52 µg/g [IQR < 20–181 µg/g] vs. 20 µg/g [IQR < 20–50 µg/g], p = 0.001).

3.5 FC in patients with IBD

Of the 91 patients ultimately diagnosed with IBD, 40 (44%) had CD, 41 (45%) had UC and 10 (11%) had IBDU. There was no significant difference in FC between the three subtypes of IBD (p = 0.56). Within the group with CD, there were 10 (25%) with L1 (ileal) disease, including one patient with L1 + 4, 18 (45%) with L2 (colonic) disease and 12 (30%) with L3 (ileocolonic) disease. FC was significantly higher in those with L2 or L3 disease, with a median (IQR) of 1280 (714–2295) µg/g than in those with L1 disease where median (IQR) FC was 495 (288–822) µg/g (p = 0.009) (Supplementary Fig. 1).

Within the group with UC, there were 3 (7%) with E1 disease (proctitis), 12 (29%) with E2 disease (left-sided colitis) and 21 (51%) with E3 disease (extensive colitis). In the remaining 5 patients, the disease extended beyond the point of insertion of the sigmoidoscope and complete staging of extent was not achieved during the initial diagnostic period. There was no significant difference in FC by disease extent when those without complete staging were excluded (p = 0.25 by Kruskal–Wallis test; Supplementary Fig. 2).

Across all patients diagnosed with IBD, there was no significant association between time to diagnosis and faecal calprotectin.

Table 1. Demographics of study population.

Variable		All n (%) or median (IQR)	Primary analysis cohort n (%) or median (IQR)
Sex	Female	627/968 (64.8%)	581/895 (64.9%)
Age at calprotectin/years		33.3 (25.7–41.0)	33.1 (25.6–40.7)
Smoking status at calprotectin	Current	204/641 (31.8%)	183/594 (30.8%)
	Ex	72/641 (11.2%)	68/594 (11.4%)
	Never	365/641 (56.9%)	343/594 (57.7%)
	Unknown	327/968 (33.8%)	301/895 (33.6%)
Drugs at calprotectin	NSAIDS	22/769 (2.9%)	0/701 (0.0%)
	Antibiotics	50/769 (6.5%)	0/701 (0.0%)
	Laxatives	16/769 (2.1%)	12/701 (1.7%)
	Opiates	39/769 (5.1%)	38/701 (5.4%)
	Immunosuppressants	82/769 (10.7%)	61/701 (8.7%)
	Loperamide	6/769 (0.8%)	2/701 (0.3%)
	Aminosalicylates	47/769 (6.1%)	42/701 (6.0%)
	Acetaminophen	1/769 (0.1%)	0/701 (0.0%)
	Aspirin	73/769 (9.5%)	52/701 (7.4%)
	Corticosteroids	2/769 (0.3%)	0/701 (0.0%)
	Unknown	199/968 (20.6%)	194/895 (21.7%)
Family history	None[a]	862/968 (89.0%)	794/895 (88.7%)
	UC	22/968 (2.3%)	21/895 (2.3%)
	CD	27/968 (2.8%)	26/895 (2.9%)
	IBDU	63/968 (6.5%)	60/895 (6.7%)
	Coeliac disease	11/968 (1.1%)	11/895 (1.2%)
	Colon cancer	14/968 (1.4%)	13/895 (1.5%)
Previous medical history	None[a]	920/968 (95.0%)	868/895 (97.0%)
	Inflammatory disease (non-gastrointestinal)	30/968 (3.1%)	24/895 (2.7%)
	Ankylosing spondylitis	30/968 (3.1%)	24/895 (2.7%)
	HIV	3/968 (0.3%)	2/895 (0.2%)
	Alcoholic liver disease	9/968 (0.9%)	0/895 (0.0%)
	Severe intercurrent illness	6/968 (0.6%)	1/895 (0.1%)

NSAIDS: non-steroidal anti-inflammatory drugs; UC: ulcerative colitis; CD: Crohn's disease; IBDU: inflammatory bowel disease unclassified.a
It has been assumed for this table that in the absence of any recorded previous medical history or family history in the patient records that there is none.

Table 2. Faecal calprotectin, age and time from calprotectin to diagnosis by diagnostic category.

Diagnosis category	n (%)	% female	Median age/years (IQR)		Median faecal calprotectin/µg/g (IQR)		Median time from calprotectin to diagnosis/days (IQR)	
Functional	566/895 (63.2%)	68.40%	32.7	(26.0–40.3)	20	(< 20–50.0)	95	(40–190)
IBD	91/895 (10.2%)	51.60%	29.8	(24.2–39.7)	1251	(532.5–2325.0)	7	(0–64)
Abnormal GI tract	65/895 (7.3%)	53.80%	37.7	(26.1–44.4)	50	(20.0–145.0)	92	(41–206)
Other GI	63/895 (7.0%)	65.10%	35	(27.0–42.8)	20	(< 20–70.0)	92	(35–153)
Other organic	32/895 (3.6%)	68.80%	31	(25.3–41.4)	22.5	(< 20–86.2)	106	(34–192)
Lost to Fup	29/895 (3.2%)	62.10%	35.8	(26.5–43.2)	135	(35.0–325.0)		
None	1/895 (0.1%)	100.00%	20.8		1825			
Symptoms resolved — no GI pathology	48/895 (5.4%)	62.50%	34.3	(25.3–42.7)	35	(< 20–76.2)		

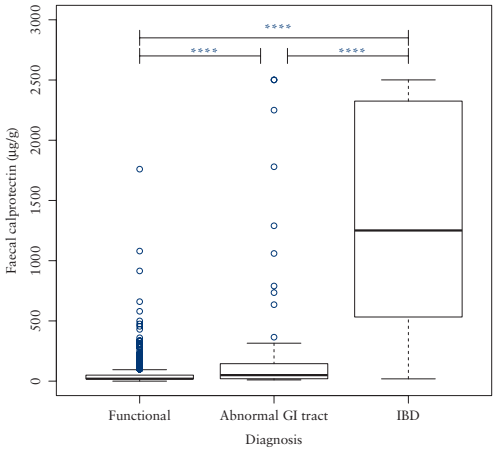


Figure 2. Box plot showing difference in faecal calprotectin between patients with functional diagnoses and those with IBD and other conditions associated with an abnormal GI tract.

3.6 Diagnostic accuracy of FC compared to other clinical parameters

Receiver operating characteristic (ROC) analysis revealed an area under the curve (AUC) for FC of 0.85 for prediction of conditions with an abnormal GI tract (including IBD) vs. functional disease, and 0.97 for prediction of IBD vs. functional disease (Fig. 3). This was significantly higher than that seen for CRP, albumin, ESR or white cell count in both cases ($p < 0.001$ for all comparisons). The sensitivities, specificities, and positive and negative predictive values for faecal calprotectin can be seen in Table 3 at different thresholds. Summaries of the number of available tests, medians and interquartile ranges for each parameter can be seen in Supplementary Table S2.

3.7 Synergistic effect of FC sampling and alarm symptoms

Alarm symptoms were present in 25% (140/566) of those ultimately diagnosed with functional disease, 86% (78/91) of those diagnosed with IBD and 54% (35/65) of those diagnosed with another condition associated with an abnormal GI tract ($p < 0.0001$). The positive predictive value of alarm symptoms for IBD or an abnormal GI tract vs. functional disease was 0.45 (95% CI 0.38–0.51), and the negative predictive value was 0.91 (0.88–0.93) with a sensitivity of 0.72 (0.65–0.79) and specificity of 0.75 (0.71–0.79). For prediction of IBD vs. functional disease, the PPV was 0.36 (0.29–0.43) and NPV was 0.97 (0.95–0.98), with a sensitivity of 0.86 (0.76–0.92) and specificity of 0.75 (0.71–0.79).

As can be seen in Table 4, FC is helpful in improving the prediction of an abnormal GI tract or IBD compared with alarm symptoms alone. Within the cohort with functional disease or an abnormal GI tract, none of the 329 patients with no alarm symptoms and a FC of < 50 µg/g was found to have IBD, while 11/36 (31%) of patients with no alarm symptoms and a FC of ≥ 200 were found to have IBD.

Thirteen patients had no alarm symptoms and a FC of < 50 µg/g, but were found to have a disease associated with an abnormal GI tract. These were 1 case of appendicitis, 1 coeliac disease, 3 with colonic adenomatous polyps, 1 with diverticulosis, 3 with GI infections (1 *Fasciola hepatica*, 1 giardiasis, 1 presumed infection with response to metronidazole), 2 with gastro-oesophageal reflux disease and 2 non-specific bowel inflammation. One of these patients with non-specific bowel inflammation was initially thought to have CD but had non-specific changes on her index colonoscopic biopsies and subsequently normal colonoscopy and biopsies.

3.8 Multivariable analysis

Multiple logistic regression analysis of predictors of IBD vs. functional disease showed that elevated FC, elevated CRP, male sex, alarm symptoms and albumin were independently significant. Age at FC and white cell count were not (Table 5).

Comparing different strategies of investigation (Table 6) demonstrated that FC alone provided the optimum specificity for both IBD vs. functional disease and IBD or abnormal GI tract vs. functional disease. The optimal combination of sensitivity and specificity was attained using the approach of alarm symptoms or FC ≥ 50 µg/g. Sensitivity and specificity for IBD vs. functional disease were 1.00 and 0.54 with this strategy, while for IBD or abnormal GI tract vs. functional disease they were 0.96 and 0.55. Adding CRP to this combination had minimal effect on sensitivity, while reducing specificity.

3.9 Low FC in patients diagnosed with inflammatory bowel disease

Three patients had a low FC (< 50 µg/g) and were diagnosed with inflammatory bowel disease. All three had alarm symptoms (two had blood in their stool and one had weight loss). Two of these patients were diagnosed with ulcerative proctitis which has not extended further in > 4 years of follow-up. One had mild terminal ileal CD with no subsequent progression.

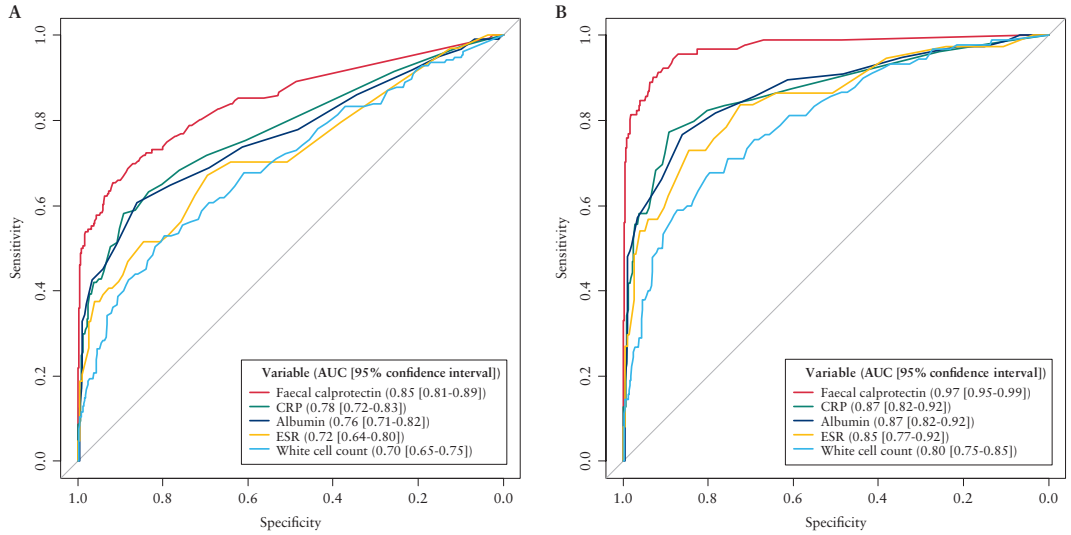


Figure 3. Receiver operating characteristic curves for calprotectin, CRP, albumin, ESR and white cell count as predictors of inflammatory bowel disease (IBD) or abnormal gastrointestinal tract versus functional disease (A) and IBD versus functional disease (B).

Table 3. Diagnostic accuracy of fecal calprotectin at different thresholdsPPV: positive predictive value; NPV: negative predictive value; PLR: positive likelihood ratio;CI: confidence interval.

Threshold fecal calprotectin (µg/g)	Sensitivity (95% CI)		Specificity (95% CI)		PPV (95% CI)		NPV (95% CI)		PLR (95% CI)	
A: Inflammatory bowel disease (IBD) or abnormal GI tract vs. functional disease										
20	0.89	(0.83–0.93)	0.49	(0.44–0.53)	0.32	(0.28–0.37)	0.94	(0.91–0.96)	1.73	(1.57–1.91)
50	0.79	(0.71–0.85)	0.74	(0.70–0.77)	0.45	(0.39–0.52)	0.93	(0.90–0.95)	3.02	(2.57–3.54)
70	0.73	(0.65–0.80)	0.80	(0.76–0.83)	0.50	(0.44–0.57)	0.92	(0.89–0.94)	3.66	(3.03–4.43)
100	0.70	(0.62–0.77)	0.87	(0.84–0.90)	0.60	(0.52–0.67)	0.91	(0.89–0.93)	5.42	(4.27–6.87)
B: IBD vs. functional disease										
20	0.99	(0.93–1.00)	0.49	(0.44–0.53)	0.24	(0.20–0.28)	1.00	(0.98–1.00)	1.92	(1.77–2.09)
50	0.97	(0.90–0.99)	0.74	(0.70–0.77)	0.37	(0.31–0.44)	0.99	(0.98–1.00)	3.70	(3.20–4.27)
70	0.97	(0.90–0.99)	0.80	(0.76–0.83)	0.44	(0.37–0.51)	0.99	(0.98–1.00)	4.84	(4.09–5.74)
100	0.96	(0.89–0.99)	0.87	(0.84–0.90)	0.54	(0.46–0.62)	0.99	(0.98–1.00)	7.41	(5.96–9.22)

Table 4. Pre- and post-test probabilities when combining alarm symptoms and fecal calprotectin.

	Pre-test probability	Post-test probability for different values of fecal calprotectin (µg/g)				
		< 20	20–49	50–99	100–199	200 +
A: inflammatory bowel disease (IBD) or abnormal GI tract vs. functional disease						
Alarm symptoms	0.45	0.15	0.18	0.24	0.50	0.91
No alarm symptoms	0.09	0.03	0.06	0.12	0.20	0.41
B: IBD vs. functional disease						
Alarm symptoms	0.36	0.02	0.05	0.05	0.41	0.89
No alarm symptoms	0.04	0.00	0.00	0.00	0.06	0.33

3.10 Cost effectiveness of FC: reducing the number of invasive investigations

Between 2005 and 2008, our practice evolved with increasing use of FC and reduction in the percentage of these patients subsequently undergoing invasive investigation. In the 2005, 63 patients underwent stool analysis for FC with 84.1% of them undergoing either sigmoidoscopy or colonoscopy. In 2008, 409 patients had stool sent for FC with 56.7% subsequently undergoing invasive investigation (Table S3).

Over the study period, 581/895 (64.9%) patients presented without alarm symptoms. 395 of these (68.0%) had a FC of < 50 µg/g. 150 of these patients (38%) had a subsequent colonoscopy and 50 (13%) a flexible sigmoidoscopy, identifying incidental adenomatous polyps in 3 patients and no other significant pathology. If the low FC had been used to triage these patients to a non-invasive approach, this would have saved £88,233 over that time period.

4. Discussion

This study uses the largest, 'real-world' population of undiagnosed patients to determine the best way of using FC at first presentation to the GI clinic to differentiate non-invasively between organic and functional disease. This allows identification of those in need of efficient and effective further investigation. Incorporating FC into the standard work-up of patients presenting with lower GI symptoms may potentially relieve pressure on hospital services by identifying patients who can be managed solely in primary care.

Our findings corroborate existing data showing that FC reliably distinguishes between patients with functional disease and IBD. Von Roon et al.'s meta-analysis of adult patients demonstrated a sensitivity of 95% and specificity of 91% when using a 50 µg/g cut-off point for differentiating IBD patients from healthy controls.[13] At the same cut-off, our study found 95% sensitivity but only 75% specificity. This agreement in sensitivity reinforces the diagnostic ability of FC in identifying patients with IBD in a large cohort of patients. The lower specificity seen in our study may be due to the patient population used, all of which have presented to services with GI symptoms, unlike the healthy control population used by Von Roon et al. Van Rheenen et al's more recent meta-analysis of six adult studies found a pooled sensitivity and specificity of 93% and 96% respectively.[22] However, inconsistent FC thresholds were used in these six studies, with 47.7% of included patients analysed using a cut-off greater than 100 µg/g, and this may have influenced the specificity.

Both ESR and CRP are markers that are commonly used to identify systemic inflammation in patients with IBD-like symptoms. In accordance with previous research, we show CRP and ESR are raised in patients with organic disease and IBD.[4,23] ROC analysis demonstrates however, that FC is superior to CRP and ESR in the diagnosis of IBD — a finding that agrees with the recent economic report produced by the NHS Centre for Evidence Based Purchasing.[24] Furthermore, we demonstrate that the NPV of FC in patients presenting with no alarm symptoms is superior to the NPV of CRP for both organic GI disease and IBD. Cost savings could be made by solely checking FC in patients presenting with lower GI symptoms, rather than checking CRP and ESR in these patients

One of the most clinically relevant findings from our data is the NPV for IBD of 99.0% when a FC threshold of 50 µg/g is used. When FC less than 50 µg/g is combined with the absence of alarm symptoms, NPV is 100.0% for IBD. This allows the exclusion of IBD from the differential diagnosis of these patients. Furthermore, in patients meeting these criteria, NPV for any GI tract abnormality is 96.1%. Of the 13 patients with no alarm symptoms and FC less than 50 µg/g who had a diagnosis of abnormal GI tract, colonoscopy was helpful in only four patients and these (diverticular disease and colonic polyps) were likely incidental findings. Clinicians can therefore be reassured that referral for colonoscopy will not identify severe organic disease in patients in whom no abnormalities are found in initial investigations. This finding could potentially be applied to a primary care scenario and aid selection of patients for colonoscopy.

With the Department of Health pricing a single colonoscopy in adults at £563 there is great potential for FC to aid more cost-effective decision making with regard to further investigation.[19] Von Rheenen et al.'s meta-analysis demonstrated that screening with FC could reduce unnecessary colonoscopies by 67% in those suspected of having IBD.[22] Similar results were documented by Mindemark and colleagues, with a reduction of colonoscopies by 50% using the FC cut off of < 50 µg/g and 67% using a FC cut off of < 100 µg/g.[25] During the study period of the present study, if patients with a FC < 50 µg/g and no alarm symptoms had not undergone lower GI endoscopy there could have been 150 fewer colonoscopies and 50 fewer flexible sigmoidoscopies. Our data reflects real world practice, with proportionally fewer patients being investigated by colonoscopy as our knowledge and experience of FC increased. A reducing trend in the numbers of those patients investigated with colonoscopy can clearly be seen as the number of FC assays received by the labs increase over the three years. The number of potential colonoscopies saved quoted above may even be more than this had our unit not been internally evaluating FC's use in clinical practice. Furthermore, the numbers we have analyzed only include patients who attended the GI clinic and had a FC sample sent. These findings could be applied to all patients who attend the GI clinic with lower GI symptoms, potentially reducing further the number of colonoscopies and resulting in even greater cost savings. It is important to take into consideration that this study uses patients referred to hospital GI services, and by virtue of this the spectrum of symptoms seen in this population is more severe when compared to all the patients presenting to GPs with GI symptoms. In primary care, FC could identify the

Table 5. Multiple logistic regression of predictors of inflammatory bowel disease vs. functional disease.

CRP: C-reactive protein.

Variable	Odds ratio (95% CI)	P
Fecal calprotectin≥50µg/g	65.3 (12.1–351.5)	1.1×10^{-6}
Alarm symptoms	19.5 (7.9–127.5)	3.0×10^{-6}
Albumin<40g/L	18.7 (4.1–85.4)	3.0×10^{-5}
Male sex	14.1 (3.8–52.2)	7.0×10^{-5}
CRP≥5g/L	6.9 (2.0–23.7)	0.002
Age at calprotectin		>0.05
White cell count >11×10^{9}/L		>0.05

Table 6. Comparison of different strategies for identifying IBD or abnormal gastrointestinal (GI) tract vs. functional disease.

IBD: inflammatory bowel disease; CRP: C-reactive protein.

Strategy	IBD vs. functional		IBD or abnormal GI tract vs. functional	
	Sensitivity	Specificity	Sensitivity	Specificity
Alarm symptoms only	0.85	0.73	0.76	0.74
CRP≥5g/L only	0.85	0.70	0.71	0.70
Faecal calprotectin (FC)≥50µg/g only	0.97	0.74	0.86	0.75
Alarm symptoms or CRP≥5g/L	0.99	0.50	0.89	0.51
Alarm symptoms or FC≥50µg/g	1.00	0.54	0.96	0.55
Alarm symptoms or CRP≥5g/L or FC≥50µg/g	1.00	0.39	0.97	0.39
Alarm symptoms or (CRP≥5g/L and FC≥50µg/g)	0.99	0.65	0.88	0.67

small numbers of patients with IBD, whilst excluding its presence in a large number of patients presenting with GI symptoms. Not only could this streamline the referral of appropriate patients to hospital, but it will also reduce the number of unnecessary referrals and invasive investigations. This does, however, require detailed pilot testing before any formal recommendations about the roll-out of FC into primary care can be made. Moreover, it is important that FC is used in the context of a defined protocol to ensure that it does not delay referral of patients with alarm symptoms and that consideration is given to possible false positive tests from aspirin and non-steroidal inflammatory drugs.

One of the strengths of this study is that all individuals without a definitive diagnosis or in whom a functional diagnosis had been made without colonoscopy were re-reviewed three years later to identify any possible latent cases of IBD or other GI disease.

This study clarifies important, clinically relevant information about FC. Awareness of the high negative predictive value of FC allows clinicians to effectively exclude IBD as a cause for gastrointestinal symptoms in patients with FC levels under 50 μg/g. FC can thus be used as an adjunct to other presenting complaints and investigations, allowing the risk stratification of patients presenting with gastrointestinal symptoms in a cost-effective manner.

Statement of interests

NAK is funded by a research training fellowship from the Wellcome Trust[grant 097943] and has had financial support to attend meetings from Warner Chillcott, Shire, MSD and Norgine. He has had speaker fees from MSD and Warner Chilcott.

JS has had research funding from Abbvie, speaker fees from Ferring and travel support from Shire.

IDRA has been on advisory boards for MSD, Hospira and P&G.

CWL has been on advisory boards for and had lecture fees from Abbvie, Hospira, MSD, Vifor, Pharmacosmos and P&G.

Acknowledgments

Guarantor

Dr Charlie Lees is the guarantor of this article.

Authorship

CWL had the initial concept and managed the study. AC, AW, NAK, JCWC, FFS, MM collected the data. WGB and KK provided the calprotectin data and biochemical expertise. IDRA and CWL reviewed cases where the diagnosis was uncertain. NAK conducted the statistical analyses. NAK, AC and AW wrote the initial draft of the manuscript. All the authors contributed to revision of the manuscript and approved the text.

Appendix A Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.crohns.2014.07.005.

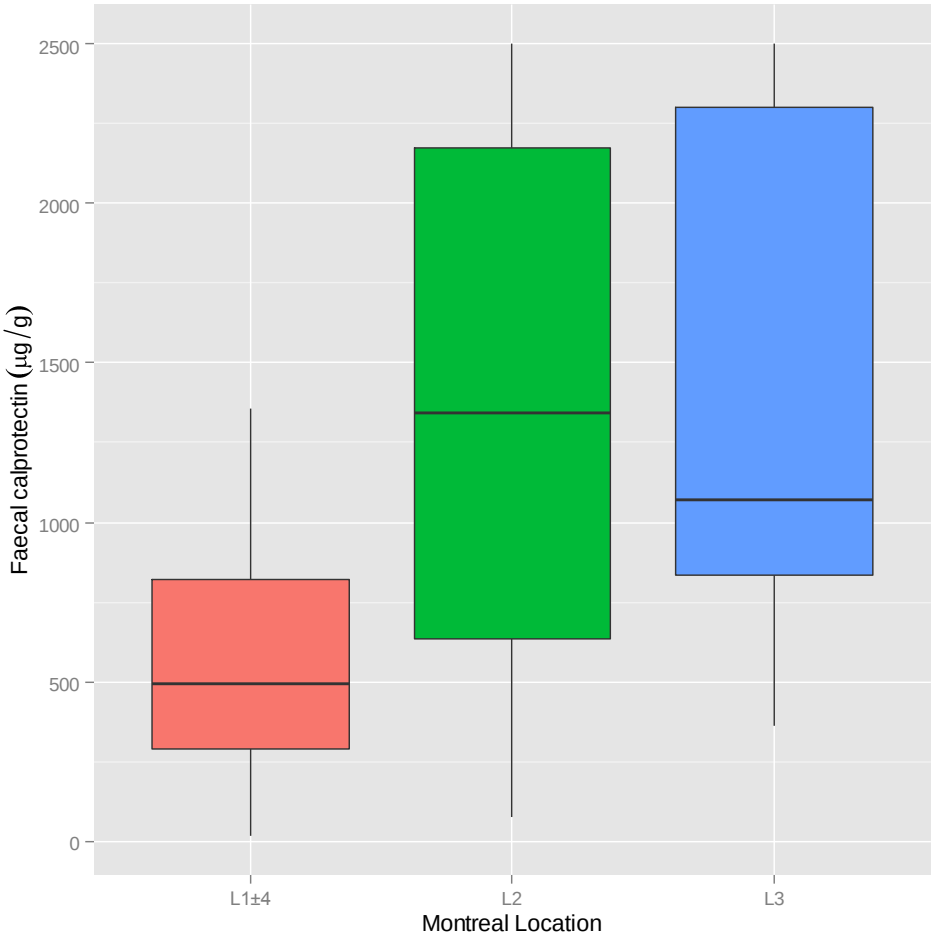
References

1. Gisbert J.P., McNicholl A.G. Questions and answers on the role of faecal calprotectin as a biological marker in inflammatory bowel disease. *Dig Liver Dis* 2009;**41**:56–66.
2. Konikoff M.R., Denson L. Role of fecal calprotectin as a biomarker of intestinal inflammation in inflammatory bowel disease. *Inflamm Bowel Dis* 2006;**12**:524–534.
3. Tibble J., Teahon K., Thjodleifsson B., Roseth A., Sigthorsson G., Bridger S., *et al.* A simple method for assessing intestinal inflammation in Crohn's disease. *Gut* 2000;**47**:506–513.
4. Schoepfer A.M., Trummler M., Seeholzer P., Seibold-Schmid B., Seibold F. Discriminating IBD from IBS: comparison of the test performance of fecal markers, blood leukocytes, CRP, and IBD antibodies. *Inflamm Bowel Dis* 2008;**14**:32–39.
5. Bonnín Tomàs A., Vila Vidal M., Rosell Camps A. Fecal calprotectin as a biomarker to distinguish between organic and functional gastrointestinal disease. *Rev Esp Enferm Dig* 2007;**99**:689–693.
6. Carroccio A., Iacono G., Cottone M., Di Prima L., Cartabellotta F., Cavataio F., *et al.* Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from irritable bowel syndrome: a prospective study in adults and children. *Clin Chem* 2003;**49**:861–867.
7. D'Incà R., Dal Pont E., Di Leo V., Ferronato A., Fries W., Vettorato M.G., *et al.* Calprotectin and lactoferrin in the assessment of intestinal inflammation and organic disease. *Int J Colorectal Dis* 2007;**22**:429–437.
8. Otten C.M.T., Kok L., Witteman B.J.M., Baumgarten R., Kampman E., Moons K.G.M., *et al.* Diagnostic performance of rapid tests for detection of fecal calprotectin and lactoferrin and their ability to discriminate inflammatory from irritable bowel syndrome. *Clin Chem Lab Med* 2008;**46**:1275–1280.
9. Schoepfer A.M., Trummler M., Seeholzer P., Criblez D.H., Seibold F. Accuracy of four fecal assays in the diagnosis of colitis. *Dis Colon Rectum* 2007;**50**:1697–1706.
10. Mowat C., Cole A., Windsor A., Ahmad T., Arnott I., Driscoll R., *et al.* Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2011;**60**:571–607.
11. NICE Faecal calprotectin diagnostic tests to differentiate inflammatory bowel disease from irritable bowel syndrome [Internet]. [cited 2013 Mar 25]; available from http://guidance.nice.org.uk/DT/12 2013.
12. Waugh N., Cummins E., Royle P., Kandala N.-B., Shyangdan D., Arasaradnam R., *et al.* Faecal calprotectin testing for differentiating amongst inflammatory and non-inflammatory bowel diseases: a systematic review and economic [Internet]. 2013; 1–241. [Available from http://www.nice.org.uk/nicemedia/live/13789/64011/64011.pdf]
13. Von Roon A.C., Karamountzos L., Purkayastha S., Reese G.E., Darzi A.W., Teare J.P., *et al.* Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* 2007;**102**:803–813.
14. Van Rheenen P.F., Van de Vijver E., Fidler V. Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis. *BMJ* 2010;**341**:c3369
15. Limburg P.J., Ahlquist D.A., Sandborn W.J., Mahoney D.W., Devens M.E., Harrington J.J., *et al.* Fecal calprotectin levels predict colorectal inflammation among patients with chronic diarrhea referred for colonoscopy. *Am J Gastroenterol* 2000;**95**:2831–2837.
16. Silverberg M.S., Satsangi J., Ahmad T., Arnott I.D., Bernstein C.N., Brant S.R., *et al.* Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005;**19 Suppl. A**:5–36.
17. Lennard-Jones J.E. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989;**170**:2–6[discussion 16–9]
18. Drossman D.A. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 2006;**130**:1377–1390.
19. Department of Health 2012–13 tariff information spreadsheet [Internet].[cited 2013 Mar 25]; available fromhttp://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_132654 2012
20. Newcombe R.G. Two-sided confidence intervals for the single proportion: comparison of seven methods. *Stat Med* 1998;**17**:857–872.
21. Simel D.L., Samsa G.P., Matchar D.B. Likelihood ratios with confidence: sample size estimation for diagnostic test studies. *J Clin Epidemiol* 1991;**44**:763–770.
22. Van Rheenen P.F. Role of fecal calprotectin testing to predict relapse in teenagers with inflammatory bowel disease who report full disease control. *Inflamm Bowel Dis* 20121–8.
23. Desai D., Faubion W.A., Sandborn W.J. Review article: biological activity markers in inflammatory bowel disease. *Aliment Pharmacol Ther* 2007;**25**:247–255.

24. Pearson S., Whitehead S., Hutton J. Evidence review: value of calprotectin in screening out irritable bowel syndrome [Internet]. Cent. Evidence-based Purch.[cited 2013 Mar 25]; available fromhttp://nhscep.useconnect.co.uk/CEPProducts/Catalogue.aspx?reportId=147 2010

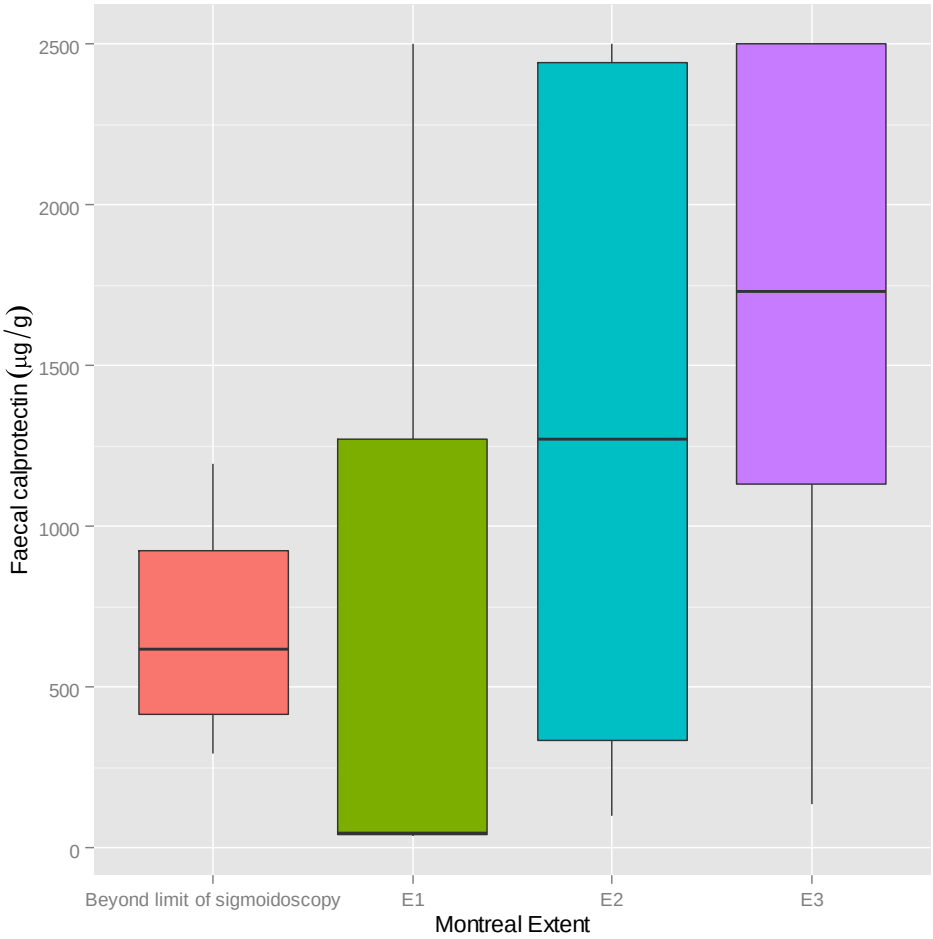
25. Mindemark M., Larsson A. Ruling out IBD: estimation of the possible economic effects of pre-endoscopic screening with F-calprotectin. *Clin Biochem* 2012;**45**:552–555.

Supplementary Figure 1: Faecal calprotectin by Montreal disease location in those with Crohn's disease



L1±4: ileal ± upper gastrointestinal involvement; L2: colonic; L3: ileocolonic

Supplementary Figure 2: Faecal calprotectin by Montreal disease extent in those with ulcerative colitis



E1: proctitis; E2: left-sided disease (distal to splenic flexure); E3: proximal to splenic flexure

6.3 Discussion of paper

This paper confirmed, in a large cohort, that faecal calprotectin measured at the point of referral to secondary care is a useful biomarker to distinguish organic intestinal disease, particularly inflammatory bowel disease, from other pathologies, particularly irritable bowel syndrome. It out-performed other commonly available blood-based markers, such as C-reactive protein, and demonstrated excellent diagnostic accuracy. One of the key strengths of our study was a long period of follow-up, to reduce the possibility of latent inflammatory bowel disease being missed.

7 Association Between Level of Fecal Calprotectin and Progression of Crohn's Disease

7.1 Introduction to paper

Building on the work I undertook on faecal calprotectin at diagnosis (see chapter 6) and the strong associations previously described between faecal calprotectin and intestinal inflammation in Crohn's,[50,52–54] , I wanted to explore how well faecal calprotectin measured during disease monitoring predicted subsequent disease progression. I built a database to collect detailed longitudinal data on Crohn's patients, and worked alongside others to populate this with phenotype data on over 900 patients, with a total of 4218 patient-years of follow-up. I hypothesised that higher baseline faecal calprotectin would be associated, independent of symptoms, with an increased rate of progression to stricturing and penetrating disease, hospitalisation and surgery.

7.2 Contributions

I conceived the study under the supervision of Charlie Lees and Ian Arnott. I designed the database and worked with Charlie Lees to co-ordinate data collection. I planned and performed the data analysis, in discussion with Charlie Lees, Gareth Jones and Nik Plevris. I wrote the manuscript, with input from Gareth Jones, Nik Plevris and Charlie Lees.

Published as: Kennedy NA, Jones GR, Plevris N, Patenden R, Arnott ID, Lees CW. Association Between Level of Fecal Calprotectin and Progression of Crohn's Disease. Clin Gastroenterol Hepatol. 2019 Feb 14. pii: S1542-3565(19)30180-6. Available from: https://dx.doi.org/10.1016/j.cgh.2019.02.017.

Accepted after peer review as Kennedy NA, Jones G-R, Plevris N, Patenden R, Arnott ID, Lees CW. Association Between Level of Fecal Calprotectin and Progression of Crohn's Disease. Clin Gastroenterol Hepatol 2019; Epub ahead of print Feb 14, 2019. Available from doi:10.1016/j.cgh.2019.02.017.

Association Between Level of Fecal Calprotectin and Progression of Crohn's Disease

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Abstract

Background & Aims

Mucosal healing is associated with improved outcomes in patients with Crohn's disease (CD), but assessment typically requires ileocolonoscopy. Calprotectin can be measured in fecal samples to determine luminal disease activity in place of endoscopy—this measurement is an important component of the treat to target strategy. We investigated whether levels of fecal calprotectin associate with subsequent CD progression.

Methods

We performed a retrospective study of 918 patients with CD (4218 patient-years of follow-up; median, 50.6 months; interquartile range [IQR], 32.8–76.0 months) managed at a tertiary medical center in Edinburgh, United Kingdom, from 2003 through 2015. Patients were included if they had 1 or more fecal calprotectin measurement made 3 months or more following their diagnosis. We collected clinical data and fecal calprotectin measurements and analyzed these data to identify factors associated with a composite outcome of progression in Montreal behavior, hospitalization, and resection.

Results

Increased level of fecal calprotectin at index visit was associated with subsequent progression of CD, independent of symptoms or disease location. The median level of fecal calprotectin at the index visit was 432 μg/g (IQR, 1365–998 μg/g) in patients who reached the composite endpoint vs 180 μg/g (IQR, 50–665 μg/g) in patients who did not. In multivariable analysis, a cutoff of 115 μg/g calprotectin identified patients who met the endpoint with a hazard ratio on of 2.4 (95% CI, 1.8–3.1; P<.0001).

Conclusion

In a retrospective analysis of patients with CD, we found that measurements of fecal calprotectin made during routine monitoring can identify patients at risk for disease progression, independent of symptoms or disease location. It is therefore important to screen asymptomatic patients for mucosal inflammation and pursue complete resolution of inflammation.

Keywords

IBD; biomarker; prognostic factor; non-invasive

Introduction

Crohn's disease (CD), a form of inflammatory bowel disease (IBD), is characterized by relapsing episodes of intestinal inflammation and the accumulation of irreversible digestive damage. Prognosis is highly variable between individuals,[1] such that the identification of patients at greatest risk of poor outcomes is an urgent research priority. Some clinical phenotypes, such as disease location and environmental factors such as smoking, have been clearly associated with poorer outcomes.[2,3] However, accurate prediction remains difficult. Over the past decade, there has been a paradigm shift away from treating until symptom resolution and towards mucosal healing as persistent subclinical bowel inflammation leads to poorer outcomes.[4–8] However this has typically required ileocolonoscopy, which is invasive, expensive and carries risk for patients.[9]

Fecal calprotectin (FC) has become well-established as a biomarker of intestinal inflammation. Calprotectin is a 36.5 kDa protein that constitutes 60% of the contents of granules in neutrophils.[10] Its use as a screening test to distinguish IBD from irritable bowel syndrome is well-supported by multiple studies, with an AUROC of 0.95 in meta-analysis.[11] Several groups have demonstrated that FC correlates well with endoscopic measures of disease activity.[12–16] There has been greater uncertainty of its role in small bowel CD, but more recently FC has been shown to correlate well with both MRI[17] and capsule endoscopy findings.[18,19]

The use of FC as a prognostic marker has been demonstrated in the context of medically- and surgically-induced remission.[20–22] In both contexts, baseline FC predicts disease flare over a follow-up period of two years, though there is also a rise notable in FC 3-4 months prior to clinical disease flare. The recent CALM study has demonstrated the effectiveness of a treat to target strategy incorporating FC in Crohn's disease.[23] However, it has still not yet

been demonstrated whether elevations in FC, irrespective of clinical symptoms, are associated with disease progression. This information would provide further support to the principle of treating beyond symptoms.

We aimed to use a large, extensively-phenotyped cohort of CD patients followed over time to determine the value of FC to predict progression of disease. We focused on endpoints associated with digestive damage: progression of Montreal behaviour[24], surgical resection or hospitalization for severe flare.

Methods

This was a retrospective cohort study of CD patients managed at the Western General Hospital, Edinburgh, UK, a teaching hospital that cares for secondary- and tertiary-referred patients with IBD. The primary inclusion criteria were a diagnosis of CD and at least one FC more than three months post-diagnosis. The *a priori* primary endpoint was a composite of progression in Montreal luminal disease behavior (B1 to B2/B3 or B2 to B3), hospitalization for flare and resectional surgery. These individual components were also defined as separate secondary endpoints. In order to reduce the possibility of merely measuring the FC at the time of the disease flare that caused the endpoint, any events that happened within 90 days after the index FC were regarded as having already happened and were not included in the endpoint analysis.

We obtained FC data from the Edinburgh FC Registry (EFCR), a record of every FC done in Edinburgh since its introduction in 2003. Patients in this initial cohort had their first FC between 2003 and 2014 and were followed up until 2015. Fecal calprotectins were requested as part of routine monitoring and also directed by patients' symptoms. These

data represent a convenience sample, and include all patients tested during that period who met our inclusion criteria.

We matched these data to existing research and clinical databases to identify patients with a known diagnosis of CD. We then interrogated the electronic and paper medical records to obtain information on demographics, symptoms, disease location and behavior over time, hospitalizations, surgical procedures, investigations and drug therapy. Disease location and behavior were classified according to the Montreal classification.[24] Changes in disease behavior were defined as occurring when the first investigation that demonstrated the change was performed, for example an MRI scan showing stricturing small bowel disease.

Patients were regarded as symptomatic either by Harvey Bradshaw Index (HBI) > 4 and/or by physician global assessment of active symptomatic luminal disease[25]. Each of the previous medical therapies was categorized as having ever taken versus never, with immunomodulators defined as azathioprine, mercaptopurine and methotrexate. Data were stored in a Microsoft Access 2003 database (Microsoft, Redmond, WA, USA).)

FC collection kits were given to patients and samples returned to the hospital biochemistry laboratories either directly or via their GP practice (samples forwarded the same day). Upon arrival at the laboratories samples are stored at -20 °C. FC was measured using a standard enzyme-linked immunosorbent assay (ELISA) technique (Calpro AS, Norway). All assays were performed utilizing the same protocol in the Department of Clinical Biochemistry at the Western General Hospital, Edinburgh. The manufacturer's reference range for distinguishing inflammatory bowel disease from functional gut disorders is >50 μg/g.

Statistical analysis was done using R 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria). The Mann Whitney U test was performed for continuous non-parametric data,

while Fisher's exact tests were done for categorical data. Survival analysis was performed using Kaplan Meier and Cox proportional hazards models.[26] For the survival models, we have reported the outcome as the proportion with maintained digestive health, i.e. the inverse of our primary endpoint. Patients were excluded from the specific analysis of progression in Montreal behavior if they were already B3 at baseline.

FC was analyzed using log-transformed data and using a predefined threshold of 250 μg/g. The optimum threshold for FC on survival analysis was then explored by examining the p values of the likelihood ratio test and the Akaike Information Criteria for Cox proportional hazard models. Variable selection for multivariable models was done using a stepwise backwards method based on Akaike Information Criterion. We performed Cox proportional hazards analyses of the effect of drug therapy up to 3 months pre or 6 months post fecal calprotectin on the primary outcome; for this analysis, patients who had disease progression within the first six months or who were censored in that period were excluded from analysis. The multistate transition data for disease progression in the overall cohort was done using the empirical transition matrix method.[27]

The principal analysis was done using the first FC for each patient where there was more than one. Owing to the retrospective nature of this dataset, these were not taken at uniform intervals. Exploratory analysis of multiple FCs was performed using the median for each rolling six-month period centered on each month following diagnosis and stratified by progression in Montreal behavior. FCs were excluded from this analysis where the patient was symptomatic at the time of sampling.

This study was conducted as a service evaluation using data collected routinely as part of clinical care, and therefore following guidance from the UK Health Research Authority did not require specific ethical approval or consent.

Results

We identified 918 CD patients meeting our inclusion criteria (Figure 1). 61.1% were female, and median age at the index FC measurement was 40.7 years (interquartile range [IQR] 28.5-54.8) (Table 1). Median follow-up time was 50.6 months (IQR 32.8-76.0), with a total of 4218 patient-years of follow-up across the cohort. At diagnosis, 81% had an inflammatory (B1) phenotype, 12% stricturing (B2) and 8% penetrating (B3). By 30 years post-diagnosis, the proportions of B1, B2 and B3 were estimated as 29%, 36% and 36% respectively (Figure 2). FC was significantly higher in patients with L3 (median 315[IQR 90 – 866] µg/g) and L2 disease (median 289 [IQR 69 – 909] µg/g) than in those with L1 disease (median 180 [IQR 65 – 445 µg/g]; $p<0.0001$).

Demographic and biomarker data on the cohort stratified by whether the patients reached the composite endpoint or not are shown in table 2. On univariable cox proportional hazards analysis, FC was strongly associated with an elevated risk of reaching the primary endpoint (Table 3), with a hazard ratio (HR) of 1.79 (95% CI 1.50 – 2.14, $p = 1.9\times10^{-10}$) for $\log_{10}$(FC). The only other blood tests nominally associated with FC on univariable analysis were CRP (p=0.016), hemoglobin (p=0.011) and platelets (p=0.003). There were also associations with younger age at diagnosis (p=0.010), female sex (p=0.021), prior immunomodulator use (p=0.012), symptoms at index visit ($p=1.2\times10^{-7}$). Smoking status, previous intestinal resection, previous anti-TNF and time period of FC measurement (pre/post 2008) use were not associated with the primary endpoint, nor was there a significant difference in the time since diagnosis at the index FC.

On multivariable Cox proportional hazards analysis, disease progression was independently associated with elevated FC, female sex, younger age, ileal/ileocolonic disease, previous immunomodulator use and symptoms (Table 3).

A further analysis was performed to explore the effect of changes in treatment before and after measurement of calprotectin (Supplementary Table 1). This was restricted to patients who did not have disease progression and were not censored within the first six months. There were no significant associations with changes in medication in the three months leading up to the measurement of fecal calprotectin. Use of steroids in the six months following calprotectin was significantly associated with disease progression (HR 1.5 [95% CI 1.16 - 2.03], p=0.003). However, this was no longer significant in a multivariable analysis that also included the FC result (Supplementary Table 2).

Above a threshold FC of 250 μg/g, the hazard ratio for reaching the primary endpoint was 1.9 (95% CI 1.5 – 2.3, $p = 5.5\times10^{-8}$, figure 3A). Using analysis of different thresholds of FC (Supplementary Figure 1), the most significant difference in progression to the primary composite endpoint with a cut-point of 115 μg/g (figure 3B) yielding a hazard ratio on multivariable analysis of 2.4 (95% CI 1.8 – 3.1, $p = 7.2\times10^{-10}$). Differences in progression were seen in all three principal Montreal locations (L1, L2 and L3; Supplementary Figure 2), in all three secondary endpoints (Supplementary Figure 3) and independent of symptom status at the index visit (Supplementary Figure 4).

Using the Kaplan-Meier estimates, the positive predictive value of an index FC >115 μg/g was 28%, 43%, 52% and 59% at 2, 4, 6 and 8 years respectively. The negative predictive value of an index FC ≤115 μg/g was 88%, 80%, 74% and 65% at 2, 4, 6, and 8 years respectively.

In a sensitivity analysis by quartiles of time from diagnosis to first fecal calprotectin, the association between calprotectin and disease progression was seen for quartiles 2 to 4, but not for the patients in the first quartile; these patients had 3 to 15.5 months between diagnosis and first fecal calprotectin (Supplementary Figure 5).

We performed an exploratory analysis using all of the available CD FC data and excluding FC taken when patients had symptoms. This analysis included 1456 FCs from 396 patients. The rolling median FC can clearly be seen to differ between those 35/396 patients with a subsequent progression in Montreal behavior and those that did not (Supplementary Figure 6).

Discussion

This study demonstrates that elevated FC is associated with increased disease progression, both as defined by a composite primary endpoint of advance in Montreal luminal behavior, surgical resection and hospitalization and by each of these endpoints when considered individually.

Mucosal healing is recognized as a target for therapy in Crohn's disease, with poorer prognosis and a higher risk of surgery associated with increased endoscopic disease activity[4–8] There is a strong correlation between FC, endoscopic disease activity and ulcer depth.[12,28] Our data show more directly that elevated FC can be used as a marker of increased risk of progression.

Although absolute index FC levels were lower in L1 patients, FC better predicted poorer outcomes in patients with L1/L3 rather than L2 disease distribution. Patients with active colonic disease may be more likely to exhibit symptoms, and thus have earlier intervention. In contrast, patients with active ileal disease may tolerate a higher level of subclinical

inflammation, resulting in delay of treatment with a greater risk of progression and complications.

Other variables associated with an adverse outcome in our analysis included younger age, which has previously been identified as an adverse prognostic factor[1], and previous immunomodulator use which is likely to be a marker for a more aggressive prior disease course. Symptomatically active disease was associated with an increased rate of disease progression, independently of elevated FC. This validates a treat-to-target approach aiming for a combination of resolution of symptoms as well as mucosal healing, with FC a marker of the latter.

Thresholds for prediction of disease relapse have varied across the literature, influenced by the disease cohort being studied and the assay used. Several studies have identified a cut-off of 250 µg/g as being useful to distinguish active from inactive disease.[20,22,29] In the present study, the optimal separation between survival curves for progression of disease was seen using a lower threshold of 115 µg/g, suggesting that lower levels of inflammatory activity may still be associated with an adverse outcome. However, any such threshold needs to be interpreted in the context of the methods of FC extraction and measurement. For example, others have shown significant variability in FC measurement between weight-based and other methods of FC extraction and similarly when comparing ELISA kits from different manufacturers'.[30,31]

We have shown that elevated FC at any point in disease course beyond the first year correlates with poorer outcome. Previous studies have demonstrated an increase in symptomatic relapse in patients with elevation of FC;[20–22] our study further indicates that this is associated with an increase in disease progression. The CALM study has recently

demonstrated better outcomes at 52 weeks when a strategy incorporating symptoms, CRP and FC was compared with clinical disease activity alone.[32] Together, these data now clearly support a treat-to-target strategy combining a patient-reported symptom score with FC as a marker of mucosal inflammation.

Strengths of the present study include the large number of patients and duration of follow-up, with a median follow-up time following index FC of greater than four years. A clinically relevant definition of disease progression was selected *a priori*, and rich phenotype information was available. Restricting measurement of endpoints to at least 90 days after the index FC should reduce bias from measuring disease activity associated with an exacerbation that went on to cause hospital admission or surgical resection. It can also be observed that the survival curves in figures 3–6 continue to separate for many months after the index FC. This suggests that identification of mucosal inflammation at any point in patient follow up, even at relatively modest levels previously considered acceptable (i.e. FC 115-250ug/g), should warrant careful monitoring and low threshold for treatment escalation decisions.

Limitations of this study relate to its retrospective nature. FCs were not collected at fixed intervals, but as determined by the treating clinician. However, routine monitoring of FC including in asymptomatic patients was established quite early on in Edinburgh after the full roll-out of the test in 2005. The study was also performed at a single centre, which may reduce heterogeneity but at the expense of generalizability. Nonetheless, although the Western General Hospital is a referral centre, it also has a large secondary care population from the local catchment. Finally, medication data were completed as accurately as was possible, but it is possible some courses of steroids, particularly those in primary care, may have been missed. This is unlikely to have introduced any systematic bias.

In conclusion, we have shown in this study that elevated fecal calprotectin is associated with an increased risk of disease progression over time in Crohn's disease. Further studies should continue to explore the utility of repeated FC measurements, and to assess whether intervention based on FC can alter disease outcome.

Acknowledgements

Data collection for this study was also undertaken by Miriam Guy, Tom Smith, Joash T Loh, David Haunschmidt, Martina Muscat, Federica Fasci Spurio and Hazel E Drummond. Kathleen Kingstone provided expertise on fecal calprotectin. Colin L Noble, Alan G Shand and Jack Satsangi provided access to their Crohn's disease patients.

Bibliography

1. Cosnes J, Bourrier A, Nion-Larmurier I, Sokol H, Beaugerie L, Seksik P. Factors affecting outcomes in Crohn's disease over 15 years. Gut 2012;61:1140–5.
2. Lunney PC, Kariyawasam VC, Wang RR, Middleton KL, Huang T, Selinger CP, et al. Smoking prevalence and its influence on disease course and surgery in Crohn's disease and ulcerative colitis. Aliment Pharmacol Ther 2015;42:61–70.
3. Sands BE, Arsenault JE, Rosen MJ, Alsahli M, Bailen L, Banks P, et al. Risk of early surgery for Crohn's disease: implications for early treatment strategies. Am J Gastroenterol 2003;98:2712–8.
4. Peyrin-Biroulet L, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant R V, et al. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. Am J Gastroenterol 2015;110:1324–38.
5. Allen PB, Olivera P, Emery P, Moulin D, Jouzeau JY, Netter P, et al. Review article: moving towards common therapeutic goals in Crohn's disease and rheumatoid arthritis. Aliment Pharmacol Ther 2017;45:1058–72.
6. Frøslie KF, Jahnsen J, Moum BA, Vatn MH, IBSEN Group. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. Gastroenterology 2007;133:412–22.
7. Schnitzler F, Fidder H, Ferrante M, Noman M, Arijs I, Van Assche G, et al. Mucosal healing predicts long-term outcome of maintenance therapy with infliximab in Crohn's disease. Inflamm Bowel Dis 2009;15:1295–301.
8. Allez M, Lemann M, Bonnet J, Cattan P, Jian R, Modigliani R. Long term outcome of patients with active Crohn's disease exhibiting extensive and deep ulcerations at

colonoscopy. Am J Gastroenterol 2002;97:947–53.

9. Lohsiriwat V. Colonoscopic perforation: incidence, risk factors, management and outcome. World J Gastroenterol 2010;16:425–30.

10. Røseth AG, Fagerhol MK, Aadland E, Schjønsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. Scand J Gastroenterol 1992;27:793–8.

11. von Roon AC, Karamountzos L, Purkayastha S, Reese GE, Darzi AW, Teare JP, et al. Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. Am J Gastroenterol 2007;102:803–13.

12. D'Haens G, Ferrante M, Vermeire S, Baert F, Noman M, Moortgat L, et al. Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. Inflamm Bowel Dis 2012;18:2218–24.

13. Schoepfer AM, Beglinger C, Straumann A, Trummler M, Renzulli P, Seibold F. Ulcerative colitis: correlation of the Rachmilewitz endoscopic activity index with fecal calprotectin, clinical activity, C-reactive protein, and blood leukocytes. Inflamm Bowel Dis 2009;15:1851–8.

14. Schoepfer AM, Beglinger C, Straumann A, Trummler M, Vavricka SR, Bruegger LE, et al. Fecal calprotectin correlates more closely with the Simple Endoscopic Score for Crohn's disease (SES-CD) than CRP, blood leukocytes, and the CDAI. Am J Gastroenterol 2010;105:162–9.

15. Sipponen T, Savilahti E, Kolho K-L, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. Inflamm Bowel Dis 2008;14:40–6.

16. Sipponen T, Kärkkäinen P, Savilahti E, Kolho K-L, Nuutinen H, Turunen U, et al. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. Aliment Pharmacol Ther 2008;28:1221–9.

17. Pendsé DA, Makanyanga JC, Plumb AA, Bhatnagar G, Atkinson D, Rodriguez-Justo M, et al. Diffusion-weighted imaging for evaluating inflammatory activity in Crohn's disease: comparison with histopathology, conventional MRI activity scores, and faecal calprotectin. Abdom Radiol (New York) 2017;42:115–23.

18. Bar-Gil Shitrit A, Koslowsky B, Livovsky DM, Shitrit D, Paz K, Adar T, et al. A prospective study of fecal calprotectin and lactoferrin as predictors of small bowel Crohn's disease in patients undergoing capsule endoscopy. Scand J Gastroenterol 2016;5521:1–6.

19. Kopylov U, Yung DE, Engel T, Avni T, Battat R, Ben-Horin S, et al. Fecal calprotectin for the prediction of small-bowel Crohn's disease by capsule endoscopy: a systematic review and meta-analysis. Eur J Gastroenterol Hepatol 2016;28:1137–44.

20. de Suray N, Salleron J, Vernier-Massouille G, Grimaud JC, Bouhnik Y, Laharie D, et al. P274 Close monitoring of CRP and fecal calprotectin levels to predict relapse in Crohn's disease patients. A sub-analysis of the STORI study. J Crohn's Colitis 2012;6:S118–9.

21. Zhulina Y, Cao Y, Amcoff K, Carlson M, Tysk C, Halfvarson J. The prognostic significance of faecal calprotectin in patients with inactive inflammatory bowel disease. Aliment Pharmacol Ther 2016;44:495–504.

22. Diederen K, Hoekman DR, Leek A, Wolters VM, Hummel TZ, de Meij TG, et al. Raised faecal calprotectin is associated with subsequent symptomatic relapse, in children and adolescents with inflammatory bowel disease in clinical remission. Aliment Pharmacol Ther 2017;45:951–60.

23. Colombel J-F, Panaccione R, Bossuyt P, Lukas M, Baert F, Vaňásek T, et al. Effect of tight control management on Crohn's disease (CALM): a multicentre, randomised, controlled phase 3 trial. Lancet (London, England) 2017; Epub ahead of print Oct 31, 2017. Available from doi:10.1016/S0140-6736(17)32641-7.

24. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 2005;19 Suppl A:5–36.

25. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. Lancet (London, England) 1980;1:514.

26. Terry M. Therneau, Patricia M. Grambsch. Modeling Survival Data: Extending the Cox Model. New York: Springer; 2000.

27. Allignol A, Schumacher M, Beyersmann J. Empirical Transition Matrix of Multi-State Models: The {etm} Package. J Stat Softw 2011;38:1–15.

28. Goutorbe F, Goutte M, Minet-Quinard R, Boucher A-L, Pereira B, Bommelaer G, et al. Endoscopic Factors Influencing Fecal Calprotectin Value in Crohn's Disease. J Crohn's Colitis 2015;9:1113–9.

29. Dhaliwal a, Zeino Z, Tomkins C, Cheung M, Nwokolo C, Smith S, et al. Utility of faecal calprotectin in inflammatory bowel disease (IBD): what cut-offs should we apply? Frontline Gastroenterol 2015;6:14–9.

30. Labaere D, Smismans A, Van Olmen A, Christiaens P, D'Haens G, Moons V, et al. Comparison of six different calprotectin assays for the assessment of inflammatory bowel disease. United Eur Gastroenterol J 2014;2:30–7.

31. Whitehead SJ, French J, Brookes MJ, Ford C, Gama R. Between-assay variability of faecal calprotectin enzyme-linked immunosorbent assay kits. Ann Clin Biochem 2013;50:53–61.

32. Colombel JF, Panaccione R, Bossuyt P, Lukas M, Baert FJ, Vanasek T, et al. Superior Endoscopic and Deep Remission Outcomes in Adults with Moderate to Severe Crohn's Disease Managed with Treat to Target Approach Versus Clinical Symptoms: Data from Calm. Gastroenterology 2017;152:S155.

Table 1 – Baseline demographics of the cohort (n=918)

Variable		Median (IQR) / Number (%)
Sex	Female	561 (61.1%)
Age at diagnosis/years		27.4 (20.1 - 42.8)
Age at calprotectin/years		40.7 (28.5 - 54.8)
Months to first calprotectin		75.5 (15.5 - 183.8)
Year of first calprotectin		2010 (2008 - 2011) (Range 2003 – 2014)
Smoking at diagnosis	Current	229 (32.5%)
	Ex	101 (14.3%)
	Never	375 (53.2%)
Montreal location	L1±L4	289 (31.7%)
	L2±L4	328 (36.0%)
	L3±L4	288 (31.6%)
	Isolated L4	6 (0.7%)
Montreal behavior at diagnosis	B1	741 (80.7%)
	B2	106 (11.5%)
	B3	71 (7.7%)
Montreal behavior at index calprotectin	B1	564 (61.4%)
	B2	200 (21.8%)
	B3	154 (16.8%)
New medication in 3 months prior to fecal calprotectin		
Steroids		91 (9.91%)
Immunomodulator		58 (6.32%)
Anti-TNF		16 (1.74%)
Any of these		146 (15.90%)
New medication in 6 months following fecal calprotectin		
Steroids		170 (18.52%)
Immunomodulator		105 (11.44%)
Anti-TNF		47 (5.12%)
Any of these		239 (26.03%)

Table 2 Demographics and investigations at index visit stratified by whether individuals reached the composite primary endpoint of progression in Montreal behavior, surgical operation or hospitalization

Variable			Primary endpoint		P
			Not reached	Reached	
Sex	M		235 (42.4%)	105 (32.5%)	0.005
	F		320 (57.7%)	217 (67.4%)	
Age at diagnosis/years			28.2 (20.9 - 45.0)	24.7 (17.9 - 38.1)	2.3×10^{-4}
Age at calprotectin/years			41.9 (30.0 - 56.3)	38.0 (26.7 - 49.8)	2.7×10^{-4}
Months to first calprotectin			69.3 (13.4 - 183.8)	85.1 (20.0 - 189.5)	0.234
Montreal location	L1		167 (30.3%)	110 (34.5%)	1.7×10^{-4}
	L2		224 (40.6%)	88 (27.6%)	
	L3		159 (28.8%)	117 (36.7%)	
Smoker at visit	No		263 (75.1%)	142 (68.9%)	0.115
	Yes		87 (24.9%)	64 (31.1%)	
Previous resection			231 (41.6%)	146 (45.3%)	0.289
Previous immunomodulator			255 (45.9%)	166 (51.6%)	0.123
Previous anti-TNF			110 (19.8%)	68 (21.1%)	0.664
Symptomatic at index visit			195 (53.4%)	162 (78.3%)	2.4×10^{-9}
Investigation		**n**			
Fecal calprotectin (ug/g)		877	180 (50 - 665)	432 (136 - 998)	6.9×10^{-12}
CRP (mg/L)		375	7 (3 - 19)	10 (4 - 27)	0.023
ESR (mm/hr)		202	21 (11 - 36)	26 (14 - 41)	0.045
Albumin (g/L)		350	40 (36 - 43)	38 (32 - 43)	0.097
Hemoglobin (g/L) (scaled to male range)		500	148 (139 - 155)	145 (133 - 154)	0.009
WCC ($\times10^9$/L)		507	7.5 (5.9 - 9.4)	7.3 (5.8 - 9.5)	0.785
Platelets ($\times10^9$/L)		489	277 (225 - 342)	305 (249 - 377)	4.9×10^{-4}

Values shown are medians (interquartile ranges) and numbers (percentages) as appropriate. P values calculated using Mann Whitney U and Fisher's exact tests for continuous and categorical data respectively.

Table 3 Univariable and multivariable analyses using Cox proportional hazards models for time to reaching primary endpoint

Variable	Univariable		Multivariable	
	HR (95% CI)	p	HR (95% CI)	p
Sex (female)	1.31 (1.04 - 1.65)	0.021	1.66 (1.23 - 2.24)	0.001
Age at diagnosis/years	0.99 (0.98 - 1.00)	0.010		
Age at calprotectin/years	0.99 (0.98 - 1.00)	0.001	0.99 (0.98 - 1.00)	0.010
No ileal involvement (Montreal L2)	0.66 (0.51 - 0.84)	7.9×10^{-4}	0.60 (0.44 - 0.82)	0.001
Previous immunomodulator	1.32 (1.06 - 1.64)	0.012	1.39 (1.04 – 1.84)	0.024
Previous anti-TNF	1.12 (0.86 - 1.46)	0.411		
Symptomatic at index visit	2.45 (1.76 - 3.42)	1.2×10^{-7}	2.07 (1.46 – 2.93)	4.1×10^{-5}
Fecal calprotectin (ug/g)*	1.79 (1.50 - 2.14)	1.9×10^{-10}	1.49 (1.17 – 1.89)	0.001
CRP (mg/L)*	1.44 (1.07 - 1.93)	0.016		
Hemoglobin (g/L) (scaled to male range)	0.99 (0.98 - 1.00)	0.011		
Platelets (x10^9/L	1.00 (1.00 - 1.00)	0.003		

* Variable $\log_{10}$ transformed prior to use in the model. Hazard ratio is for each 10-fold increase in the variable.

HR: Hazard Ratio; CI: Confidence Interval

Figure 1 – Derivation of the cohort of patients with Crohn's disease, fecal calprotectin (FC) and follow-up data

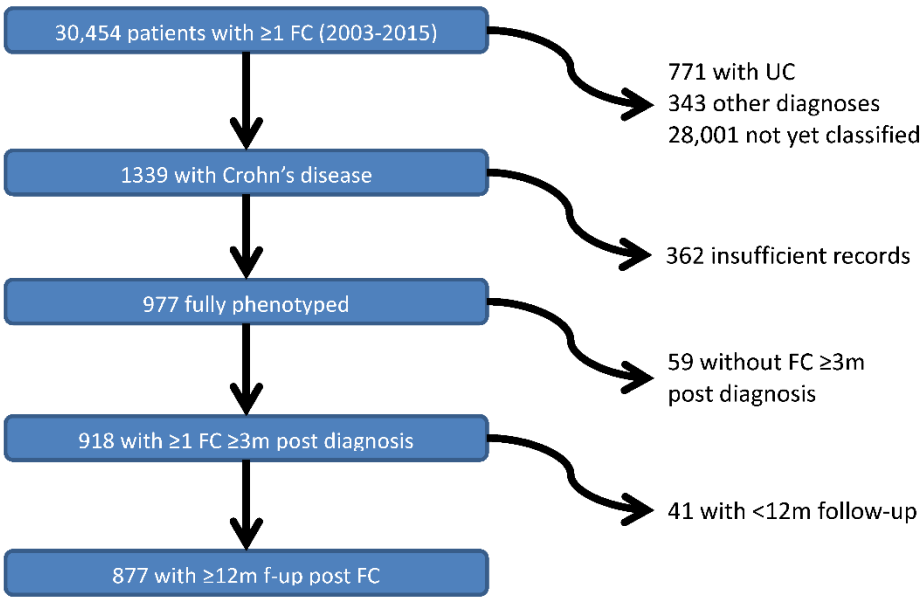


Figure 2 – Disease progression over time in the whole cohort as estimated by the empirical transition matrix method

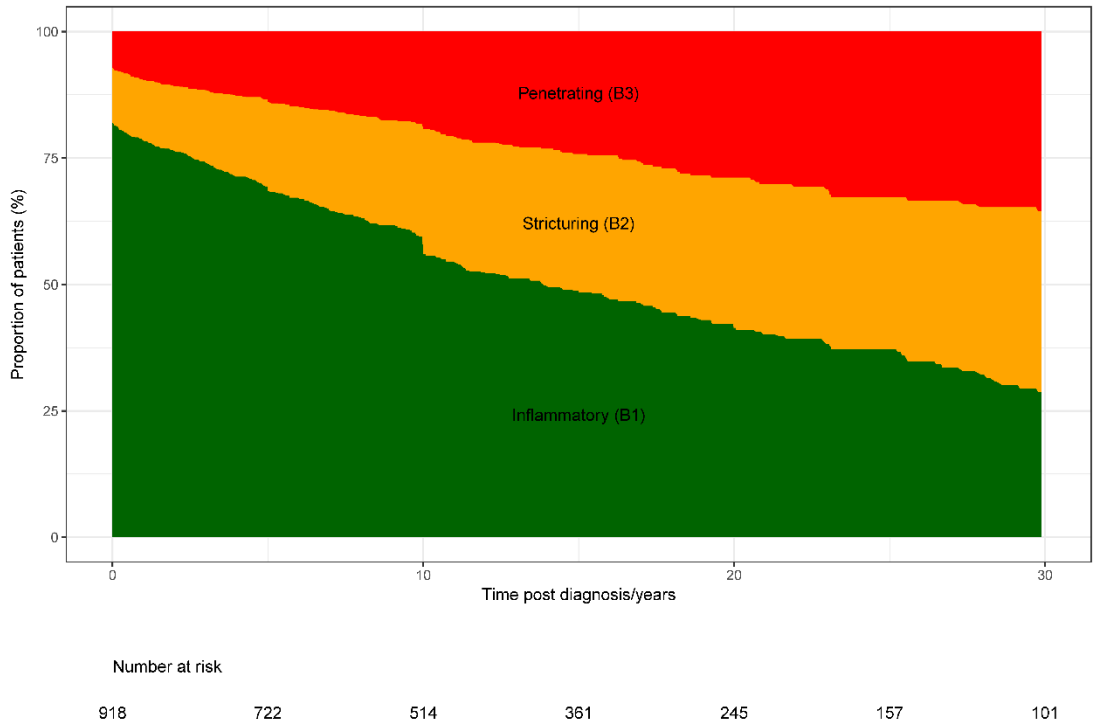
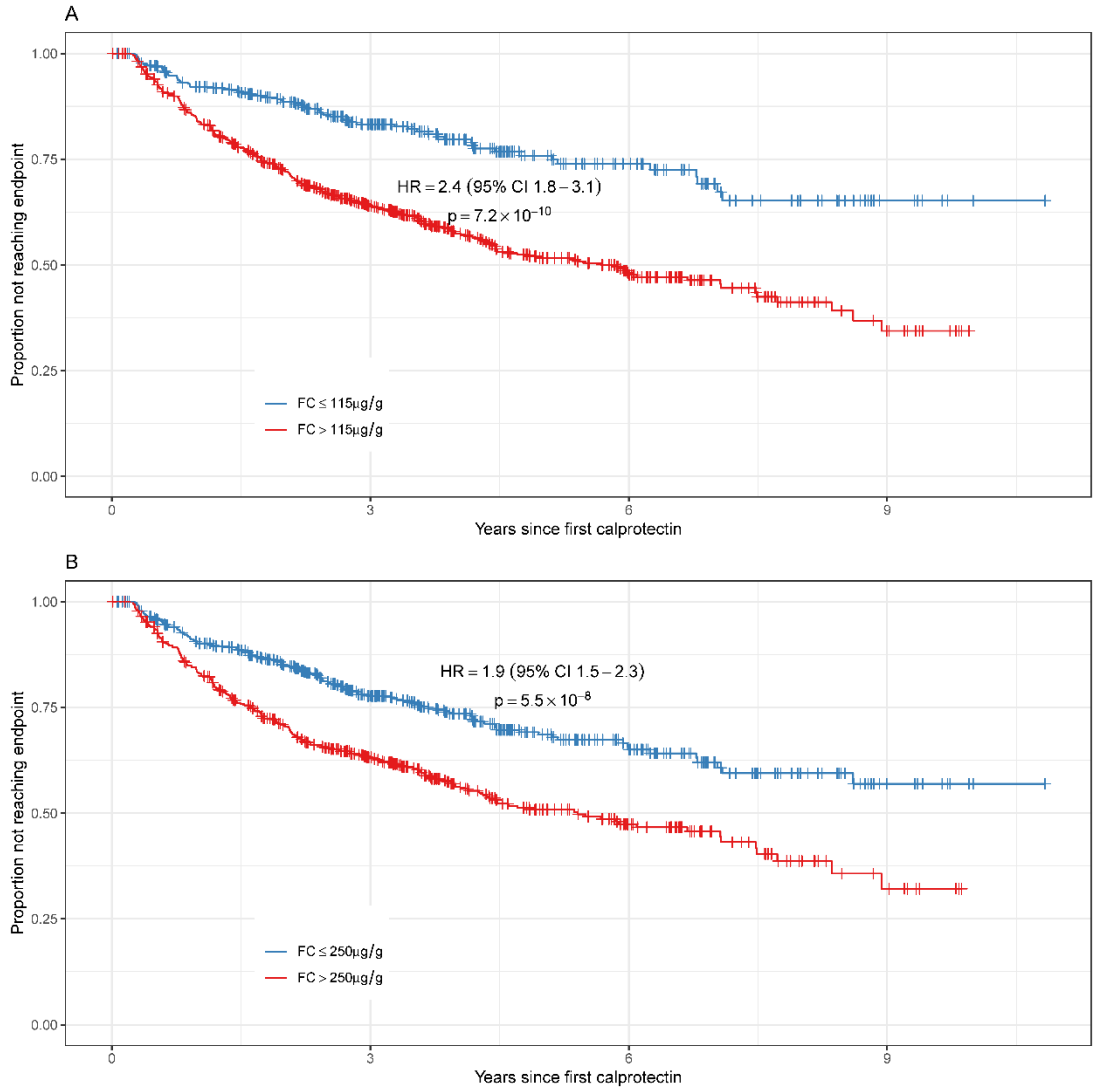


Figure 3 – Kaplan-Meier plot of time to reaching primary endpoint stratified by fecal calprotectin > 250 µg/g (A) and > 115 µg/g (B) at index visit



The outcome of maintained digestive health is defined here as the inverse of the primary study endpoint (a composite of progression in Montreal behavior, hospitalization or surgery)

Supplementary Table 1 – Univariable survival analysis of the primary composite endpoint stratified by fecal calprotectin and changes of medication 3 months before and 6 months after calprotectin; patients reaching the endpoint or censored in the first 6 months were excluded.

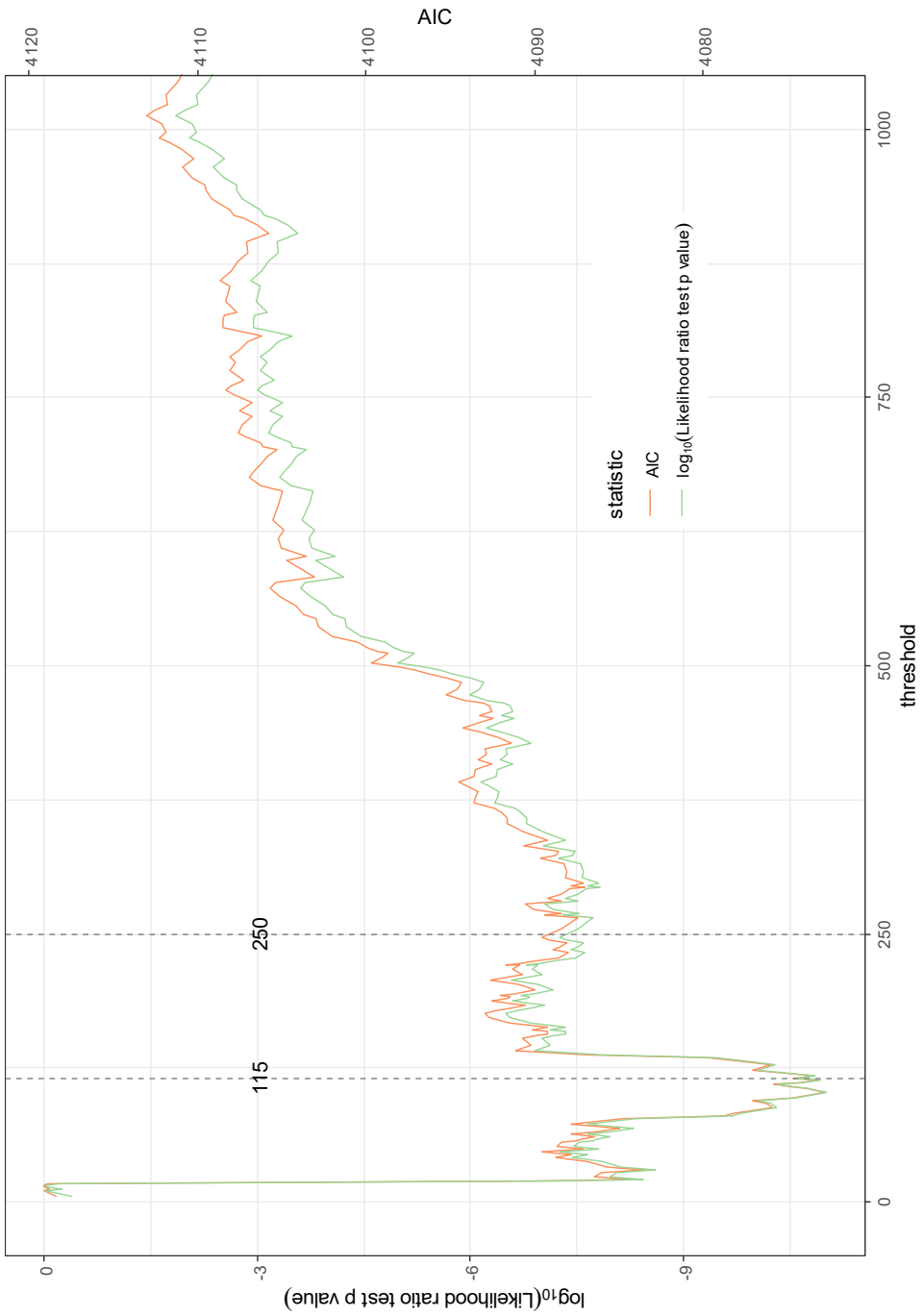
Variable	Hazard ratio (95% CI)	p
Sex (female)	1.30 (1.01 - 1.66)	0.042
Age at diagnosis/years	0.99 (0.98 - 1.00)	0.017
Age at calprotectin/years	0.99 (0.98 - 1.00)	0.001
Montreal location L2 vs (L1/L3)	0.66 (0.50 - 0.86)	0.002
Smoker	1.13 (0.82 - 1.55)	0.467
Previous resection	0.97 (0.77 - 1.23)	0.796
Previous immunomodulator	1.29 (1.02 - 1.63)	0.036
Previous anti-TNF	1.13 (0.82 - 1.57)	0.450
Symptoms or HBI	2.25 (1.58 - 3.20)	6.4×10^{-6}
Fecal calprotectin > 115 µg/g	2.36 (1.76 - 3.17)	1.1×10^{-8}
log10(Fecal calprotectin)	1.80 (1.49 - 2.19)	2.6×10^{-9}
New medication in 3 months prior to fecal calprotectin		
Steroids	0.84 (0.55 - 1.29)	0.436
Immunomodulator	1.22 (0.75 - 1.99)	0.429
Anti-TNF	1.76 (0.72 - 4.26)	0.213
Any of these	1.10 (0.79 - 1.53)	0.567
New medication in 6 months following fecal calprotectin		
Steroids	1.53 (1.16 - 2.03)	0.003
Immunomodulator	1.26 (0.88 - 1.80)	0.211
Anti-TNF	1.25 (0.73 - 2.13)	0.424
Any of these	1.48 (1.15 – 1.91)	0.002

Supplementary Table 2 – multivariable Cox proportional hazards model of the association between primary composite endpoint, fecal calprotectin and changes in medication before and after the calprotectin

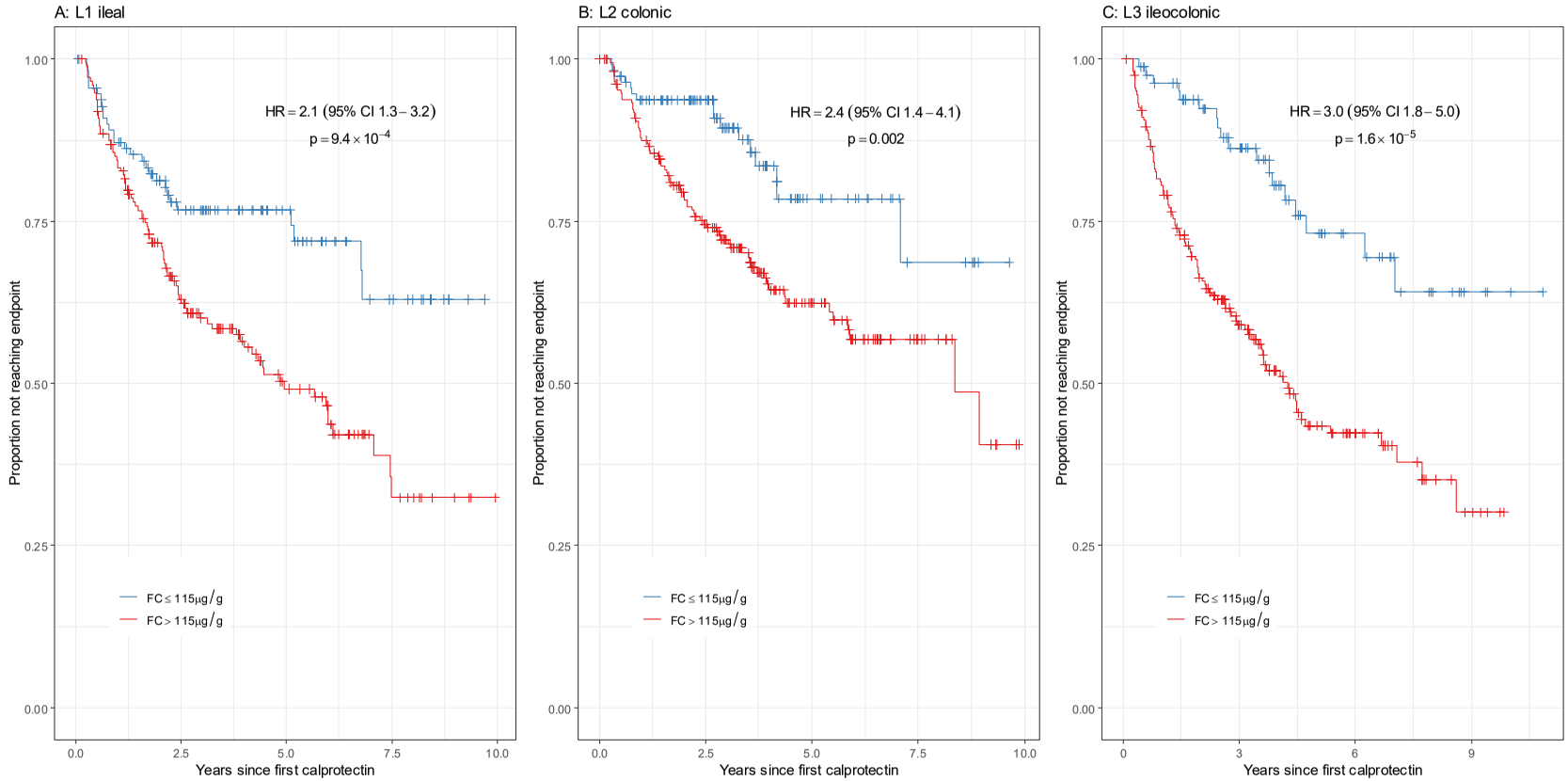
Term	Hazard Ratio (95% CI)	p
$\log_{10}$ (fecal calprotectin)	1.78 (1.45–2.19)	5.0×10^{-8}
Anti-TNF within 3 months prior	1.45 (0.58–3.61)	0.43
Immunomodulators within 3 months prior	1.15 (0.69–1.91)	0.60
Steroids within 3 months prior	0.70 (0.45–1.08)	0.11
Anti-TNF within 6 months post	0.89 (0.51–1.55)	0.69
Immunomodulators within 6 months post	0.99 (0.67–1.45)	0.95
Steroids within 6 months post	1.20 (0.88–1.62)	0.25

CI: confidence interval

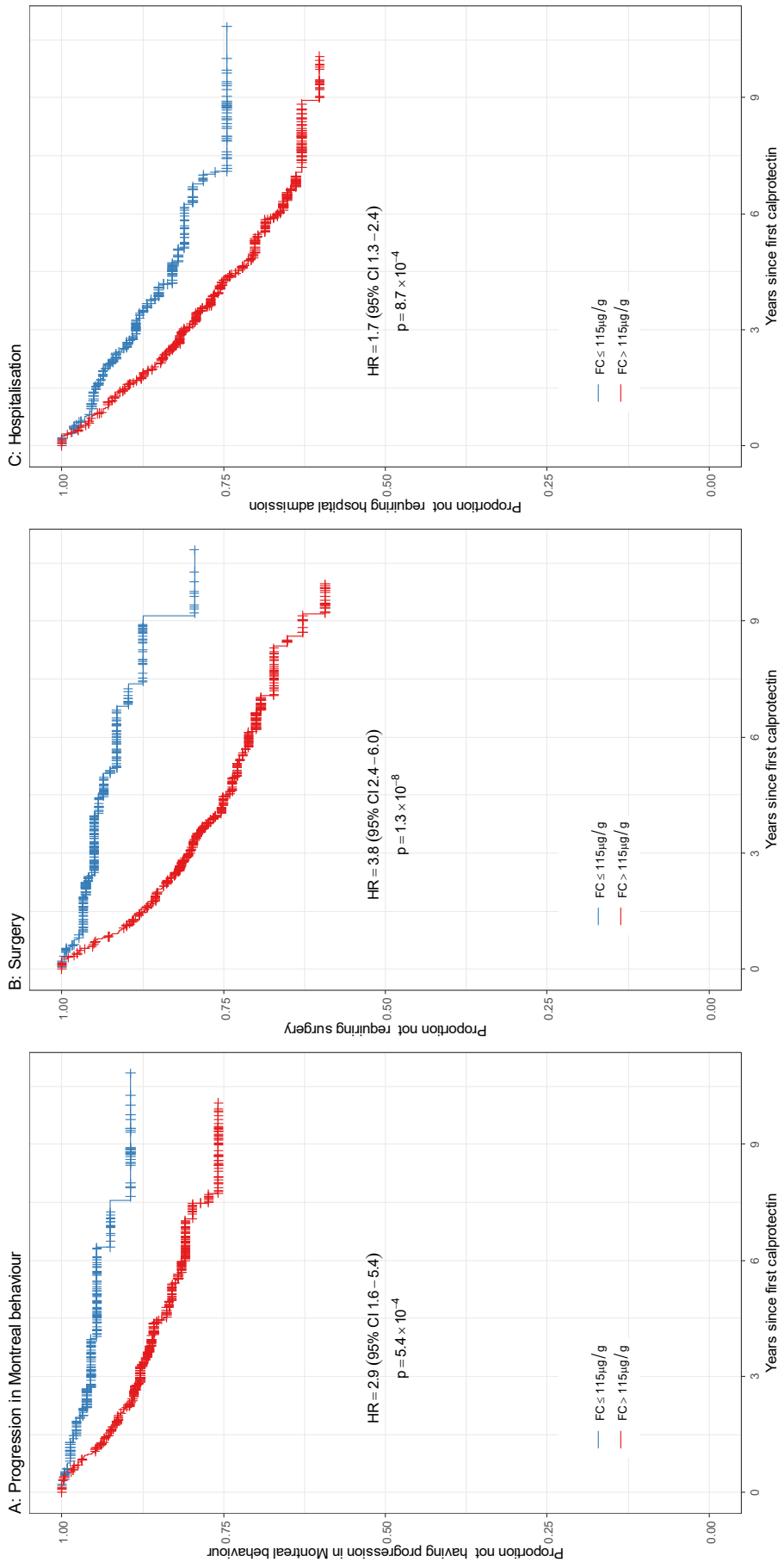
Supplementary Figure 1 – Comparison of Akaike Information Criterion and Likelihood ratio test p value for Cox proportional hazards models at different thresholds of fecal calprotectin



Supplementary Figure 2 – Kaplan-Meier plot of time to reaching primary endpoint stratified by fecal calprotectin > 115 µg/g at index visit and by Montreal location

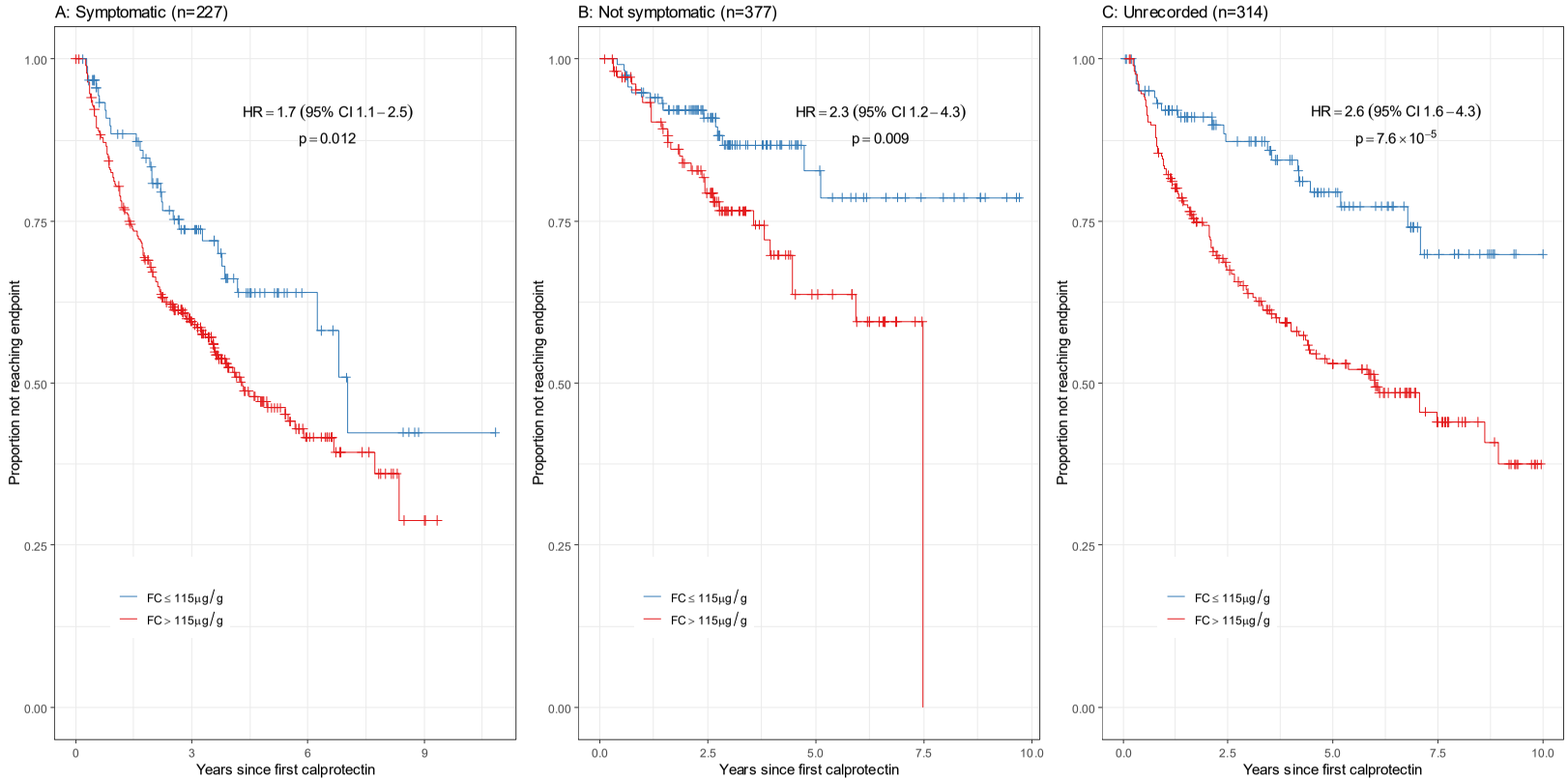


The outcome of maintained digestive health is defined here as the inverse of the primary study endpoint (a composite of progression in Montreal behavior, hospitalization or surgery)



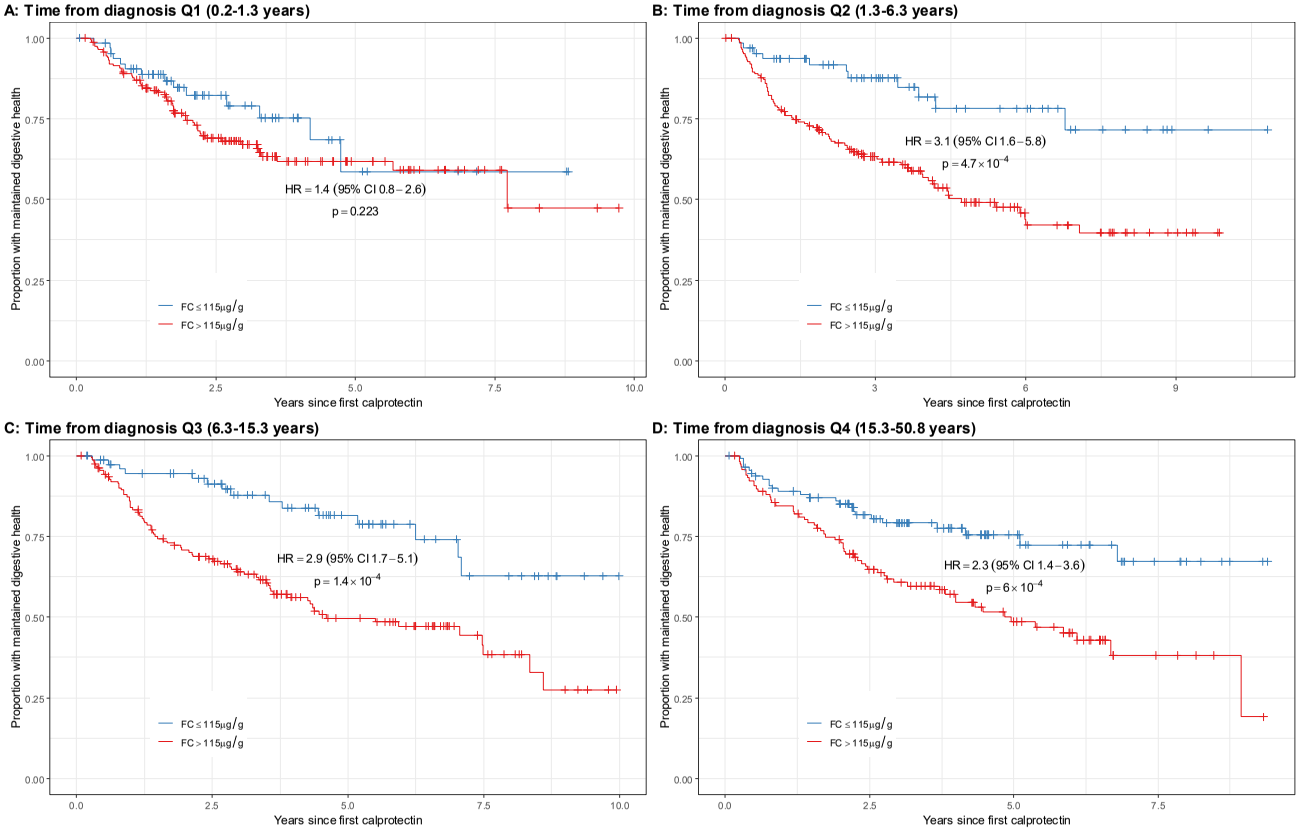
Supplementary figure 3 – Kaplan Meier plots of the three separate secondary outcome measures stratified by fecal calprotectin >115 µg/g.

Supplementary Figure 4 – Kaplan Meier plots of the primary endpoint stratified by fecal calprotectin >115 µg/g and by the presence of symptoms at the index visit.



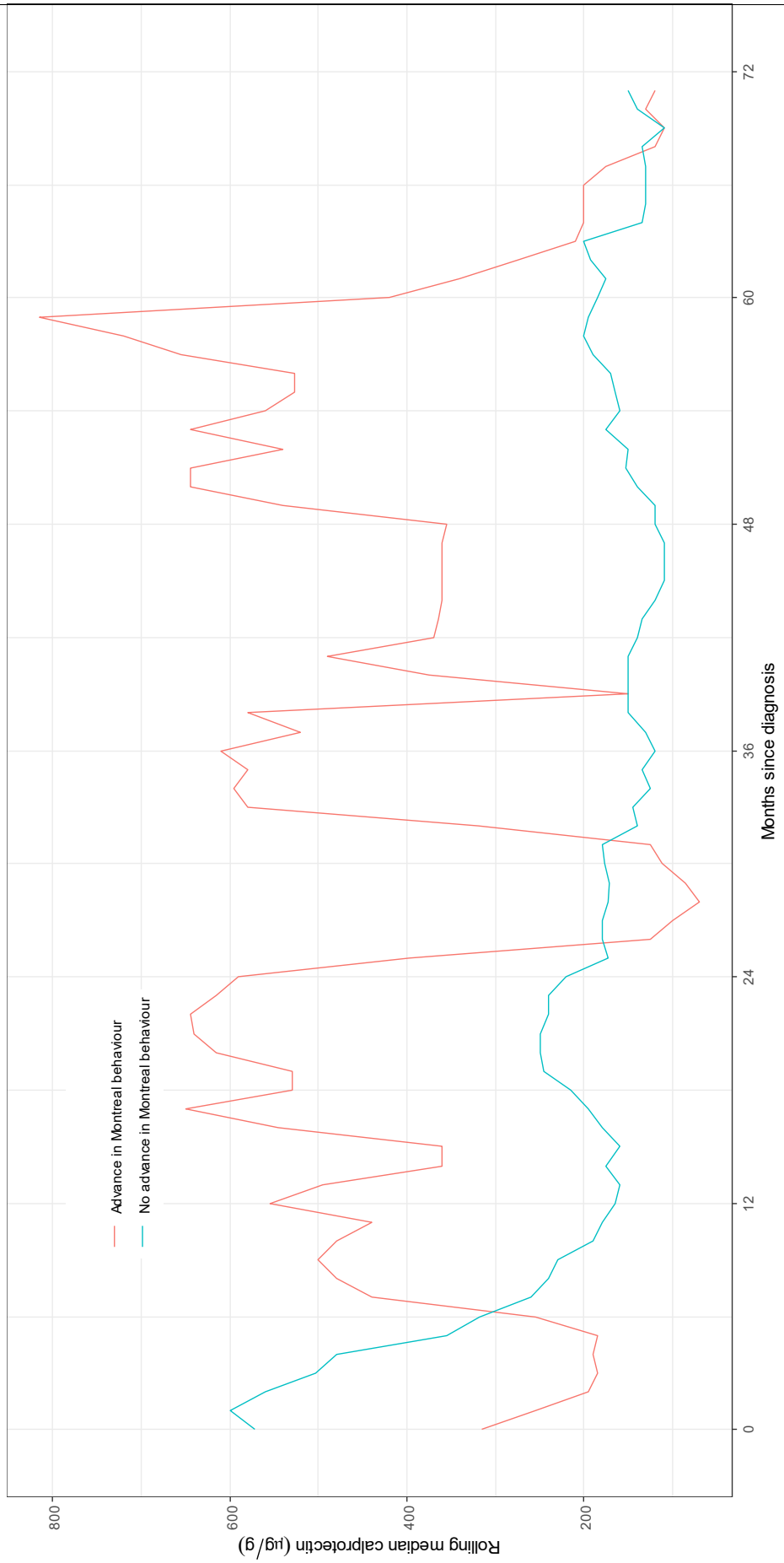
The outcome of maintained digestive health is defined here as the inverse of the primary study endpoint (a composite of progression in Montreal behavior, hospitalization or surgery)

Supplementary Figure 5 – Kaplan Meier plots of the primary endpoint stratified by fecal calprotectin >115 µg/g and by the quartile of time between diagnosis and first fecal calprotectin



The outcome of maintained digestive health is defined here as the inverse of the primary study endpoint (a composite of progression in Montreal behavior, hospitalization or surgery)

Supplementary Figure 6 – Rolling median fecal calprotectin by time since diagnosis stratified by subsequent progression in Montreal behavior



7.3 Discussion of paper

This paper bridges the gap between two previous observations. Persistent mucosal inflammation has been associated with progression of Crohn's disease,[34,138], and faecal calprotectin has been associated with the degree of mucosal inflammation.[50,52–54] Importantly, I was able to demonstrate that a single faecal calprotectin measurement has value in predicting subsequent disease course. This adds weight to the arguments for tight control of disease, and the use of faecal calprotectin as a non-invasive biomarker to guide treatment escalation.

In both this chapter and the previous one, calprotectin has been used as a non-invasive biomarker of intestinal inflammation. Calprotectin is a heterodimer of two proteins in the S100 protein family, S100A8 and S100A9. It is the most abundant cytosolic protein in neutrophils[139] and is also found in other inflammatory cells including monocytes, macrophages and squamous epithelial cells.[140] Calprotectin plays a number of roles including cytoskeletal modification, leucocyte recruitment and induction of release of pro-inflammatory cytokine.[141] It has antibmicrobial activity against a wide range of pathogens including *Escherichia coli¸ Candida albicans* and *Listeria monocytogenes.* Although generally thought of as a pro-inflammatory molecule, calprotectin may also have anti-inflammatory activity, including in the regulation of the cytokine response to lipopolysaccharide.[142]The precise role of calprotectin the disease pathophysiology of Crohn's disease is not well described, and most of the literature on calprotectin focuses on its role as a biomarker of inflammation rather than as potentially an active player in the disease process. Although most clinical use of calprotectin is within gastroenterology, there are a number of other disease areas where there is evidence for its potential as a biomarker including cystic fibrosis[143], rheumatology[136] and acute kidney injury.[134]

8 Thiopurine withdrawal during sustained clinical remission in inflammatory bowel disease: relapse and recapture rates, with predictive factors in 237 patients

8.1 Introduction to paper

Crohn's disease is a long-term condition and most patients will require maintenance therapy of some sort. If patients achieve stable remission, there are multiple reasons to consider treatment withdrawal. These include the risk of adverse events, the requirements for regular monitoring, pregnancy, cost and patient preferences. However, physicians and patients need to weigh the potential benefits of treatment cessation against the risks of relapse. This chapter details one of two large, multisite studies I undertook to better understand both the risk of relapse following stopping maintenance therapy, and factors predictive of relapse. Specifically, this study covers withdrawal of the thiopurines azathioprine and mercaptopurine.

8.2 Contributions

I conceived the study along with Charlie Lees. The data was collected by physicians and junior doctors across multiple sites around the UK. I co-ordinated data collection, aggregated the data and performed analysis. I wrote the manuscript along with Rahul Kalla and Charlie Lees.

Published as: Kennedy NA, Kalla R, Warner B, Gambles CJ, Musy R, Reynolds S, et al. Thiopurine withdrawal during sustained clinical remission in inflammatory bowel disease: relapse and recapture rates, with predictive factors in 237 patients. Aliment Pharmacol Ther 2014;40:1313–23. Available from: https://dx.doi.org/10.1111/apt.12980.

AP&T Alimentary Pharmacology and Therapeutics

Thiopurine withdrawal during sustained clinical remission in inflammatory bowel disease: relapse and recapture rates, with predictive factors in 237 patients

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Publication data
Submitted 30 June 2014
First decision 18 July 2014
Resubmitted 30 August 2014
Accepted 14 September 2014
EV Pub Online 6 October 2014

This article was accepted for publication after full peer-review.

SUMMARY

Background
Thiopurines (azathioprine and mercaptopurine) remain integral to most medical strategies for maintaining remission in Crohn's disease (CD) and ulcerative colitis (UC). Indefinite use of these drugs is tempered by long-term risks. While clinical relapse is noted frequently following drug withdrawal, there are few published data on predictive factors.

Aim
To investigate the success of planned thiopurine withdrawal in patients in sustained clinical remission to identify rates and predictors of relapse.

Methods
This was a multicentre retrospective cohort study from 11 centres across the UK. Patients included had a definitive diagnosis of IBD, continuous thiopurine use ≥3 years and withdrawal when in sustained clinical remission. All patients had a minimum of 12 months follow-up post drug withdrawal. Primary and secondary end points were relapse at 12 and 24 months respectively.

Results
237 patients were included in the study (129 CD; 108 UC). Median duration of thiopurine use prior to withdrawal was 6.0 years (interquartile range 4.4–8.4). At follow-up, moderate/severe relapse was observed in 23% CD and 12% UC patients at 12 months, 39% CD and 26% UC at 24 months. Relapse rate at 12 months was significantly higher in CD than UC ($P = 0.035$).
Elevated CRP at withdrawal was associated with higher relapse rates at 12 months for CD ($P = 0.005$), while an elevated white cell count was predictive at 12 months for UC ($P = 0.007$).

Conclusion
Thiopurine withdrawal in the context of sustained remission is associated with a 1-year moderate-to-severe relapse rate of 23% in Crohn's disease and 12% in ulcerative colitis.

Aliment Pharmacol Ther 2014; **40:** 1313–1323

doi:10.1111/apt.12980

INTRODUCTION

Thiopurines have been in clinical use for 50 years and remain the backbone of maintenance strategies for IBD, either as monotherapy or in combination with an anti-tumour necrosis factor agent.[1] Azathioprine (AZA) and its metabolite mercaptopurine (MP) are effective in maintaining clinical remission in patients with Crohn's disease (CD) and ulcerative colitis (UC).[2–8] Around 10–28% of patients report side effects (most commonly nausea) of which 50–80% will discontinue the drug as a result.[9, 10] Thiopurines have a narrow therapeutic window and carry a risk of dose-dependent myelosupression[4, 9, 11, 12] and hepatotoxicity.[10, 13] A subset of the population who carry two loss of function thiopurine methyltransferase (TPMT) alleles have the greatest risk of myelosupression and serious adverse events.[14] Continuous use of thiopurines has also been linked with malignancies such as lymphoma and non-melanoma skin cancer.[15, 16] A large prospective study of 19 486 IBD patients showed incidence rates of non-melanoma skin cancer for current and previous AZA use at 0.66/1000 and 0.38/1000 patient years respectively (age < 50) and a cumulative increase with age.[15] Beaugerie showed an incidence rate of 0.9/1000 patient years for lymphoma in those receiving AZA (n = 19 486), casting doubt on its long-term safety.[16] These long-term risks make clinicians and patients wary about indefinite use of thiopurines despite the risk of relapse on withdrawal.

The relapse rates after stopping thiopurines have been reported in CD at 21–41% at 1 year with a cumulative increase to 61–85% at 5 years.[3, 17–21] In UC, one randomised controlled trial and three retrospective studies showed relapse rates of 35–77% at 1 year and 65–75% at 5 years.[9, 22–24] However, most of these studies had patients on treatment for a short period of time (Table 1) and perhaps therefore overestimate the risk of disease relapse in patients who are in sustained clinical remission.

We aimed to examine relapse rates following thiopurine withdrawal along with predictive factors and the success of recapture in a large group of patients with at least 3 years of continuous thiopurine therapy for CD or UC.

METHODS

Study design

A retrospective multi-centre clinical audit was performed with patients identified from 11 IBD centres across the UK. Detailed case note review was performed in all patients using a standardised, pre-designed proforma. Data were collected for patient demographics including age, sex, weight, smoking status, age at diagnosis and date of diagnosis. Details of drug therapy included the type of thiopurine used, start date, initial dose and maximum dosage, age at withdrawal and any dose tapering at withdrawal, plus concomitant medications. Details of parameters at withdrawal included Montreal classification and behaviour, laboratory markers [C-reactive protein (CRP), haemoglobin, white cell count, platelets, albumin], endoscopic findings and reasons for withdrawal. Relapse was recorded including any change in drug therapy or reintroduction of thiopurines. Patients were identified by searching IBD databases and/or clinic lists of those attending out-patient IBD clinics to reduce the risk of bias from physicians recalling only those patients who had relapsed.

Inclusion criteria

Patients had a definitive diagnosis of UC or CD and continuous thiopurine use for at least 35 months. They were in clinical remission at the time of drug withdrawal as defined by physician global assessment and no use of corticosteroids within the preceding 6 months. The minimum follow-up time following withdrawal was 12 months (or moderate–to-severe relapse within 12 months). Patients were excluded if they were on concomitant anti-TNF therapy at the point of thiopurine withdrawal.

Disease relapse was defined by severity and categorised as mild, moderate or severe. Mild relapse was defined by the use of topical treatments or commencement or dose increase of oral 5-aminosalicylate (5-ASA) while moderate relapse was defined by the use of oral steroids or recommencement of thiopurine. Admission to hospital, surgery, use of intravenous corticosteroids or commencement of anti-TNF was considered a severe relapse.

At study design, primary end-point was defined as moderate-to-severe relapse at 12 months while secondary end-point was moderate-to-severe relapse at 24 months.

Statistical analysis

Data were analysed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and R 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). Continuous data are presented as medians and interquartile ranges and were analysed using a Mann–Whitney U-test. Categorical data are presented as numbers and percentages and were analysed using χ^2 or Fisher's exact tests as

Table 1 | Summary of AZA/MP withdrawal studies

Study	Design Study cohort	Number of patients studied following thiopurine withdrawal	Drug	Duration of thiopurine (months)	Follow-up (months)	Relapse rate	Factors predictive of relapse
O'Donoghue (1978)[3]	RCT CD	51	AZA	>24	6	1 year: Control 41%; AZA 5%	
Lemmann (2005)[19]	RCT CD	43	AZA	>42	18	18 months: Control 21.3%; AZA 7.9%	CRP Hb Time without steroids
Bouhnik (1996)[17]	Retrospective CD	42	AZA or MP	31 (median)	60	1 year: 38% 3 years: 61% 5 years: 75%	Male gender Age <31 years Remission<4 years
Kim (1999)[20]	Audit CD	36	MP	>6	60	1 year: 36% 2 years: 71% ≥3 years: 85%	Younger age Higher dose of 6MP during remission
Treton (2009)[18]	Open label CD	66	AZA	68 (median)	60	1 year: 14% 3 years: 52.8% 5 years: 62.7%	CRP ≥20 g/L Hb <120 g/L Neutrophils ≥4.0 × 10^9/L
Fraser (2002)[9]	Retrospective case series CD and UC	CD – 79 UC – 143	AZA	24 (mean)	60	CD/UC 1 year: 37%/37% 3 years: 66% 5 years: 75%/75%	
Sokol (2010)[31]	Audit CD	47	AZA	>42	60	2 years: 57% 5 years: 73.3%	Male Non-smoking
Hawthorne (1992)[24]	RCT UC	34	AZA	21 (mean)	12	1 year: Control 59%; AZA 35%	
Lobel (2004)[22]	Retrospective review UC	22	MP	45 (median)	40	1 year: 77% 2 years: 100%	
Cassinotti (2009)[23]	Retrospective review UC	127	AZA	47 (median)	60	1 year: 35% 3 years: 59% 5 years: 65%	Shorter duration of AZA (in remission)

appropriate. Survival analysis was done using Kaplan–Meier analysis in the survival package in R. Patients was censored at the point of most recent follow-up. Estimates of relapse rates for each severity category over time were generated from the overall survival function and the proportion of relapses of that category to that time point. For analysis of predictive factors, each factor was analysed in those patients for whom those data were available. Patients with additional reasons for withdrawal that could potentially have influenced laboratory parameters were excluded from analysis of these parameters.

Multivariable analysis was performed using Cox Proportional Hazards. Backward stepwise regression was used to select variables for the final model. Variables that did not lead to a lower Akaike information criterion (AIC) were excluded in a stepwise manner, and finally variables whose hazard ratio had a 95% confidence interval that crossed one were excluded. Continuous data were then converted to categorical data by finding the thresholds that gave the lowest AIC for the fitted model.

RESULTS

Across all centres, 264 patients were submitted. 27 were excluded, for reasons detailed in Table S1, leaving 237 patients, 129 with CD and 108 with UC, in the primary analysis (Table 2; breakdown by study centre in Table S2). The median duration of thiopurine use prior to drug withdrawal was 6.0 years (IQR 4.4–8.2) for CD and 5.8 years (IQR 4.5–8.5) for UC. The median follow-up post drug withdrawal in those without relapse was 32 months (IQR 24–51) for CD and 36 months (IQR 21–52) for UC. Median CRP was 4.0 mg/L (IQR 2.5–6.0) in CD and 2.5 mg/L (IQR 2.5–4.0) in UC (Table 3).

All patients were in sustained clinical remission at the time of thiopurine withdrawal; 35/237 (22 CD; 13 UC) had an additional trigger for drug cessation (Table S3).

Table 2 | Study demographics, Montreal classification and disease behaviour for patients in clinical remission on thiopurines

Variable	Crohn's disease, n = 129	Ulcerative colitis, n = 108
Females (%)	76 (59.8%)	42 (39.6%)
Median (IQ range) age at withdrawal/years	38 (28–48)	42 (33–58)
Current smokers (%)*	23 (19.2%)	4 (4.3%)
Median (IQR) duration thiopurine use/years	6.0 (4.4–8.2)	5.8 (4.4–8.5)
Range duration thiopurine use/years	2.9–18.7	2.9–18.0
Median (IQR) peak AZA dose/mg	125 (100–150)	150 (112–150)
Median (IQR) duration follow-up in those without relapse/months	31.7 (23.9–50.8)	36.0 (20.6–52.2)
Median year stopped AZA (range)	2008 (1980–2012)	2008 (1999–2011)
Montreal location†		
L1 ± L4	29/123 (23.6%)	
L2 ± L4	48/123 (39.0%)	
L3 ± L4	44/123 (35.8%)	
L4	2/123 (1.6%)	
Montreal behaviour†		
B1	88/123 (71.5%)	
B2	16/123 (13.0%)	
B3	19/123 (15.4%)	
Montreal extent‡		
E1		23/97 (23.7%)
E2		26/97 (26.8%)
E3		48/97 (49.5%)
5ASA at time of withdrawal	40 (31.0%)	83 (76.1%)

* Smoking status unknown in 23 patients.

† Montreal location and behaviour unknown in six patients

‡ Montreal extent unknown in 11 patients.

Thiopurines were tapered prior to withdrawal in 87 patients (37%). Data on length of taper were available in 48 of these patients, with a median duration of 12 weeks (IQR 8–26).

Disease relapse and predictive factors: univariable analysis

23% of CD patients had a moderate-to-severe relapse within 12 months of thiopurine withdrawal as compared to 12% in UC patients (Figure 1). There was a significant difference in survival without moderate-to-severe relapse between CD and UC assessed by logrank test (P = 0.035). CRP at time of drug withdrawal was associated with significantly greater relapse in CD within 12 months (P = 0.005) but was not predictive in UC (Table 4). Relapse at 12 months in CD was also associated with having tapered the thiopurine at withdrawal (P = 0.004). In the UC cohort, white cell counts at withdrawal were significantly higher in those who relapsed by 12 months (P = 0.007), although the upper quartile was still in the normal range.

Multivariable analysis

Disease location and the most significant univariable laboratory parameters (haemoglobin, white cell count and CRP) were included in multivariable models for CD and UC. The Cox proportional hazards method was used to create a model to assess the contribution of each variable to risk of relapse. After backwards stepwise exclusion of variables that did not contribute to the model, WCC and CRP remained for CD, and only WCC remained for UC (Table 5). Thresholds were then found to allow stratification of patients at higher and lower risk, and to allow creation of survival curves (Figure 2).

Consequences of relapse

Among all CD patients, by 12 months, 23 patients (18%) had required systemic corticosteroids, four patients (3%) had required anti-TNF therapy, seven patients (5%) had required hospital admission and five patients (4%) had required resectional surgery. Among all UC patients, by 12 months, six patients (6%) had required systemic steroids, one patient (1%) hospitalisation and no patient

Table 3 | Blood parameters for Crohn's disease and ulcerative colitis cohort at the time of thiopurine withdrawal

	Crohn's disease		Ulcerative colitis	
Test	Number of patients	Median (IQR)	Number of patients	Median (IQR)
Haemoglobin (g/L)*	107	150 (141–158)	94	148 (140–155)
White cell count (10^9/L)	107	6.4 (5.3–8.4)	94	6.0 (4.8–7.0)
Platelets (10^9/L)	105	266 (220–343)	92	260 (213–312)
CRP (mg/L)	81	4.0 (2.5–6.0)	65	2.5 (2.0–4.0)
Faecal calprotectin (μg/g)	6	36 (27–71)	2	71 (56–86)
Albumin (g/L)	87	43 (41–46)	73	45 (42–47)

Hb, Haemoglobin; WCC, White cell count; Plt, Platelets; CRP, C-reactive protein.

* Hb for females was scaled to male range to allow for comparison across sexes.

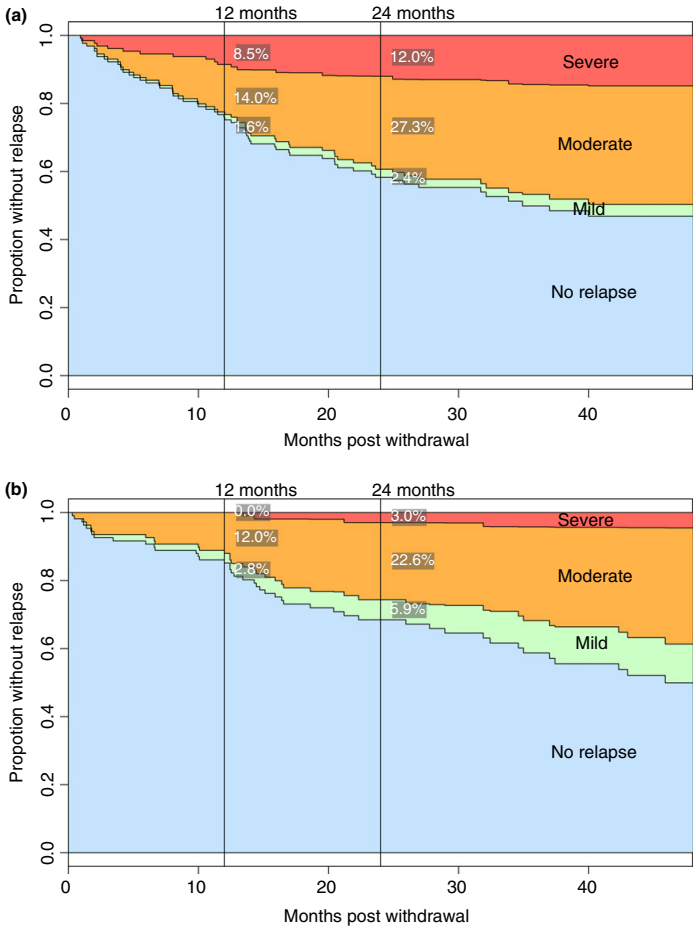

Figure 1 | Survival analysis of relapse following withdrawal of thiopurines for sustained remission of Crohn's disease (a) and ulcerative colitis (b).

required anti-TNF or resectional surgery. By the end of follow-up, a further three CD and two UC patients had required resectional surgery, although this was for dysplasia in one of the UC cases.

Within the 48 CD patients with a moderate-to-severe relapse at any point and did not require surgery or anti-TNF, 42 (88%) had a thiopurine re-introduced. Of those, reintroduction was successful in 31 (74%)

Table 4 | Factors assessed against moderate-to-severe relapse by 12 months and diagnosis

	Crohn's disease			Ulcerative colitis		
	No relapse by 12 months	Relapse by 12 months	*P*	No relapse by 12 months	Relapse by 12 months	*P*
Female sex	62/96 (64.6%)	14/29 (48.3%)	0.174	34/90 (37.8%)	6/13 (46.2%)	0.783
Smoking status at withdrawal						
Current	17/92 (18.5%)	6/27 (22.2%)	0.366	4/82 (4.9%)	0/10 (0.0%)	0.825
Ex	15/92 (16.3%)	7/27 (25.9%)		24/82 (29.3%)	4/10 (40.0%)	
Never	60/92 (65.2%)	14/27 (51.9%)	54/82 (65.9%)	6/10 (60.0%)		
Age at diagnosis	24.0 (18.3–31.8)	25.5 (19.2–35.1)	0.587	28.0 (22.5–44.2)	28.0 (19.3–41.0)	0.586
Age when starting thiopurine	29.0 (21.3–41.0)	30.0 (22.5–43.0)	0.988	36.0 (26.5–52.5)	35.0 (27.0–44.0)	0.723
Additional reason for withdrawal	19/98 (19.4%)	2/29 (6.9%)	0.156	10/92 (10.9%)	2/13 (15.4%)	0.642
Maximum dose by weight (mg/kg)	1.8 (1.5–2.2)	1.9 (1.6–2.2)	0.39	1.9 (1.7–2.1)	2.1 (2.0–2.2)	0.101
Tapered at withdrawal	27/98 (27.6%)	17/29 (58.6%)	**0.004**	34/92 (37.0%)	7/13 (53.8%)	0.387
5ASA at withdrawal	26/98 (26.5%)	12/29 (41.4%)	0.193	71/92 (77.2%)	9/13 (69.2%)	0.504
Montreal location						
L1 ± L4	25/94 (26.6%)	4/27 (14.8%)	0.096			
L2 ± L4	30/94 (31.9%)	16/27 (59.3%)				
L3 ± L4	37/94 (39.4%)	7/27 (25.9%)				
Pure L4	2/94 (2.1%)	0/27 (0.0%)				
Montreal behaviour						
B1	66/93 (71.0%)	20/28 (71.4%)	1.000			
B2	12/93 (12.9%)	4/28 (14.3%)				
B3	15/93 (16.1%)	4/28 (14.3%)				
Montreal extent						
E1				19/85 (22.4%)	4/10 (40.0%)	0.276
E2				22/85 (25.9%)	3/10 (30.0%)	
E3				44/85 (51.8%)	3/10 (30.0%)	
Haemoglobin (g/L)*	151 (145–159)	143 (139–154)	0.101	149 (139–155)	145 (140–151)	0.496
White cell count ($\times 10^9$/L)	6.2 (5.3–8.2)	7.6 (5.5–8.6)	0.270	5.9 (4.7–6.8)	7.7 (6.5–9.4)	**0.007**
Platelets ($\times 10^9$/L)	265 (220–316)	268 (226–375)	0.303	260 (213–312)	290 (250.5–324)	0.218
CRP (mg/L)	4.0 (2.1–6.0)	7.0 (3.8–16.5)	**0.005**	2.5 (2.0–4.0)	3.0 (2.8–4.5)	0.286
Albumin (g/L)	44.0 (41.0–46.0)	43.0 (41.0–45.0)	0.259	45.0 (42.2–47.0)	44.0 (41.0–45.0)	0.187

* Haemoglobin for females scaled to male range to allow comparison across sexes.

P values less than 0.05 are highlighted in bold.

Table 5 | Multivariable analysis of predictive factors for relapse following thiopurine withdrawal: final Cox proportional hazards model. (a) Crohn's disease; (b) Ulcerative colitis

Analysis as continuous variables			Analysis as categorical variables		
Variable	Hazard ratio (95% confidence interval)	*P*-value	Optimum threshold to split data	Hazard ratio when split by threshold (95% CI)	*P*-value when split by threshold
(a)					
White cell count	1.18 (1.04–1.33)	0.011	≥6.6 × 10^9/L	3.75 (1.87–7.54)	0.0002
C-reactive protein	1.04 (1.00–1.07)	0.035	≥14 g/L	3.2 (1.48–7.05)	0.003
(b)					
White cell count	1.44 (1.11–1.87)	0.007	≥9.1 × 10^9/L	6.70 (1.86–24.2)	0.004

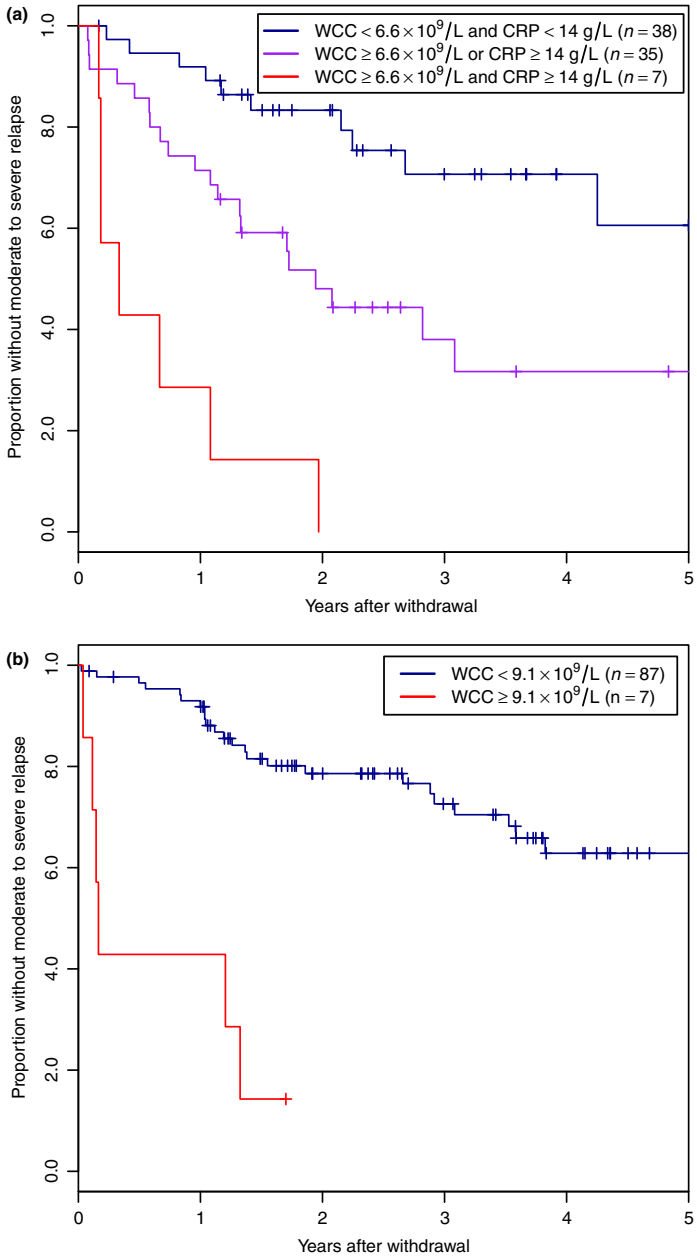

Figure 2 | Survival analysis of relapse following withdrawal of thiopurines for sustained remission stratified by predictive factors in Crohn's disease (a) and ulcerative colitis (b).

although the majority (21/31, 68%) also required systemic steroids to reinduce remission. For UC patients, thiopurines were reintroduced in 24 of 34 (71%) patients with a moderate-to-severe relapse not requiring surgery or anti-TNF. This was successful in 22 patients (92%), with 11 (50%) requiring systemic steroids also.

DISCUSSION

In patients with CD, our study shows a moderate-to-severe relapse rate of 24% at 1 year and 39% at 2 years after thiopurine withdrawal. This is similar to published series showing a relapse rate of 21–41% with a cumulative increase in the rate with time (Table 1). In addition, our study demonstrates a significant greater relapse rate in CD patients compared to UC. While this study does not address the rate of flare in those continuing therapy, a recent meta-analysis showed that the odds ratio of a flare in those stopping azathioprine versus those continuing was 0.15 [95% Confidence Interval (CI) 0.05–0.44] at 12 months and 0.30 (95% CI 0.08–1.23) at 18 months.[25]

Previous studies have shown that CRP of 20 mg/L or higher, a neutrophil count of 4.0×10^9/L, a haemoglobin (<12 g/dL), male gender, age ≤31 and duration of remission less than four years were factors predictive of relapse.[17, 18] However, duration of AZA/MP use and the definition of remission varied with each study making it difficult to compare their study outcomes. Fraser *et al.*, with $n = 222$ patients (79 CD, 143 UC), found no correlation between disease flare and clinical or laboratory indices.[9] Our study shows that CRP is highly predictive of relapse in this cohort, a finding similar to Lemann *et al.*[19] Tapering of thiopurine prior to withdrawal was also noted here to be associated with relapse, but practice with relation to tapering was quite different between the included centres and it is likely the observed differences in relapse rates relate to other, unmeasured factors rather than tapering itself.

In patients with UC, our study showed a lower relapse rate of 11% at 12 months and 21% at 24 months. This contrasts with a relapse rate after drug withdrawal in other published studies as high as 35–77% at 1 year and 65–75% at 5 years (Table 1). We used strict criteria to define sustained remission which included continuous thiopurine use for a minimum ≥3 years and subsequent withdrawal when in sustained clinical remission (absence of symptoms and no corticosteroids for >6 months). This will have impacted the subsequent relapse rates.

In the UC cohort, our study shows that a raised white cell count is highly predictive of a relapse after drug withdrawal. Hawthorne *et al.* performed a small RCT trial and found younger age to be the statistically significant predictive factor for relapse.[24] Cassinoti *et al.* performed a multicentre observational study of 127 UC patients and found that relapse during treatment with AZA, withdrawal of AZA due to drug toxicity and disease extent to be predictive of disease relapse at drug withdrawal. Patients in this study had concomitant aminosalicylates, masking the true effects of AZA.[23] On the contrary, a large single centre study with 143 UC patients did not show any factors predictive of relapse.[9]

The definition of clinical remission is important when evaluating drug withdrawal studies. Studies have used various clinical disease activity indices and laboratory markers to define clinical remission. Two randomised controlled trials used the Crohn's Disease Activity Index (CDAI),[19, 21] while others used the Harvey Bradshaw Index (HBI).[9, 17, 20] O'Donoghue *et al.* and Lobel *et al.* utilised the Physician Global Assessment (PGA) score to define remission and disease flare in CD.[3, 22] We used the PGA clinical index and corticosteroid use (in the last 6 months) to define remission.

Recapture data have only been reported by Treton *et al.* in CD patients where 22 of the 23 patients were successfully retreated with AZA.[18] Although a small cohort, our study is the only study to show retreatment success in both disease groups. However, it should be noted that 25/29 patients with CD and moderate-to-severe relapse within 12 months (20% of the overall CD cohort) required systemic steroids, anti-TNF or hospital admission and five of these patients required resectional surgery. Further large studies are needed to ascertain re-treatment success as this would have an impact on our decisions to withdraw thiopurines.

With high cumulative relapse rates after thiopurine withdrawal in sustained remission, devising a set of key relapse indicators that encompass clinical, endoscopic and laboratory markers would be beneficial. Our study highlights the importance of risk stratification in patients before considering drug withdrawal. The knowledge of these predictive factors may be translated onto the anti-TNF group of patients; however, multicentre trials are required to validate this. The STORI study, looking at infliximab withdrawal in CD remission showed that the presence of no more than two risk factors (a combination of clinical and biological markers) carried a 15% risk of relapse at 1 year.[26] Similarly, risk stratification in patients on long-term AZA/MP treatment who are risk of disease flare post drug

withdrawal can be adopted in clinical practice. Future studies could use pre-defined risk groups to assess relapse rates post drug withdrawal.

In addition to risk factors for disease relapse, adverse events with long-term use must be taken into account when considering drug withdrawal. The small but definite association of non-melanoma skin cancer and lymphoma with long-term thiopurine use has been reported.[15, 16] The risk of non-melanoma cancer is greater when treating older patients with IBD.[15] In addition, the comorbidity rate is significantly higher in the elderly group (age >65 years) with IBD.[27, 28] With an ageing population worldwide, the number of older patients with IBD is also expected to increase.[29] Therefore, treatment strategies with thiopurines would need further evaluation and a careful consideration.

The key strengths of our study are threefold. Our study is one of the largest to date looking at AZA/MP withdrawal. While many studies used varied parameters to define remission, we used strict clinical parameters with at least 3 years of continuous thiopurine use prior to drug withdrawal.

Patients within this study were selected for withdrawal by their physicians on the basis of their assessment, and so the withdrawal rates may not be generalisable to all patients in clinical remission. For example, physicians may have been less likely to withdraw patients with perianal disease or rectal disease. There were also limited data available on faecal calprotectin; it is likely that as an accurate marker of endoscopic disease activity[30] it would prove highly useful in predicting relapse in this context, as has been seen for infliximab withdrawal in the STORI study.[26] The study may have been underpowered to fully assess the predictive power of all of the factors assessed.

Thiopurines remain an integral part of disease management in IBD patients with evidence of its role in sustaining long-term remission. However, bearing in mind the side effects and risks of malignancy with long-term immunosuppression, it is crucial to identify a sub-cohort who are at highest risk of disease flare. Our study and data from the STORI trial suggest that patients in clinical and biochemical remission have a low risk of relapse. Of those who relapse after drug withdrawal, reintroduction of thiopurines allows recapture in the majority of IBD patients, particularly in UC. However, in a select group of patients (CD cohort), long-term thiopurines may be in their best interest, especially if the consequences of disease flare have an impact on morbidity and subsequent remission rates.

AUTHORSHIP

Guarantor of the article: Dr Charlie Lees.

Author contributions: NAK and CWL conceived the study. PI, JM, MP, TA, JRFC, IA, JS, AJL, MS, JOL, CWL co-ordinated data collection at their respective sites. BW, CJG, RM, SR, RD, NH, RF, SM, SMS, CAL, HAH, DG collected the data. NAK aggregated the data and performed analysis. RK wrote the initial draft of the manuscript. NAK and CWL co-ordinated revision of the manuscript as guided by all of the authors who approved the final version of the manuscript.

ACKNOWLEDGEMENTS

Declaration of personal interests: NAK has served as a speaker for Warner Chilcott, Abbvie and MSD, and has had support to attend meetings from Shire. He has research funding from the Wellcome Trust [097943].

CAL has served as a speaker for Genentech, and has received research funding from the Wellcome Trust [093885], Genentech, Techlab, Immundiagnostik and Roche tissue diagnostics.

PMI has served as a speaker, a consultant and/or an advisory board member for Abbvie, Ferring, Ferrinject, Genentech, Johnson and Johnson, MSD, Pharmacosmos, Shire, Symprove, Takeda, Tillotts and Warner-Chilcott, and has received research funding from Genentech and Theradiag.

JM has served as a consultant and an advisory board member for Genentech and Tillotts Pharmaceuticals, and has received research funding from Genentech.

TA has served as an speaker, a consultant and an advisory board member for AbbVie, MSD, Ferring, Warner Chilcott, and Napp pharmaceuticals. He has received research funding from Crohn's and colitis UK, CORE, the International serious adverse events consortium, AbbVie and MSD.

IDRA has been an advisory board member for Vifor and has had travel supported by Shire. J. Satsangi has served as a speaker, a consultant and an advisory board member for MSD, Ferring, Abbvie and Shire, and has received research funding from Abbvie.

JS has served as a speaker, a consultant and an advisory board member for MSD, Ferring Abbvie and Shire, and has received research funding from Abbvie.

MS has acted as a speaker for Warner Chilcott and the Falk Foundation, and as a consultant for Abbvie.

JOL has served as a speaker, consultant or advisory board member for Abbvie, Atlantin Healthcare, Ferring, MSD, Takeda, Shire and Warner Chilcott and received research funding from MSD, Shire and Takeda.

CWL has served as an speaker, a consultant and an advisory board member for Abbvie, MSD, Hospira, Pharmacosmos, Vifor Pharma, Dr Falk, Takeda, Warner Chilcott, and Shire, and has received research funding from Abbvie and Shire.

RK, BW, CJG, RM, SR, RD, HN, RF, RH, SM, SMS, HAH, AJL, MP, JRFC have no personal interests to declare.

Declaration of funding interests: Dr Nick Kennedy is supported by a grant from the Wellcome Trust [097943]. Dr Christopher Lamb is supported by a grant from the Wellcome Trust [093885/Z/10/Z].

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Reasons for exclusion from primary cohort.

Table S2. Numbers of included patients from each centre.

Table S3. Reasons for thiopurine withdrawal in addition to sustained remission in Crohn's disease and ulcerative colitis.

REFERENCES

1. Brooke BN, Hoffmann DC, Swarbrick ET. Azathioprine for Crohn's disease. *Lancet* 1969; **2**: 612–4.
2. Candy S, Wright J, Gerber M, *et al.* A controlled double blind study of azathioprine in the management of Crohn's disease. *Gut* 1995; **37**: 674–8.
3. O'Donoghue DP, Dawson AM, Powell-Tuck J, *et al.* Double-blind withdrawal trial of azathioprine as maintenance treatment for Crohn's disease. *Lancet* 1978; **2**: 955–7.
4. Present DH, Korelitz BI, Wisch N, *et al.* Treatment of Crohn's disease with 6-mercaptopurine. A long-term, randomized, double-blind study. *N Engl J Med* 1980; **302**: 981–7.
5. Markowitz J, Grancher K, Kohn N, *et al.* A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; **119**: 895–902.
6. Pearson DC, May GR, Fick GH, *et al.* Azathioprine and 6-mercaptopurine in Crohn disease. A meta-analysis. *Ann Intern Med* 1995; **123**: 132–42.
7. Pearson DC, May GR, Fick G, *et al.* Azathioprine for maintaining remission of Crohn's disease. *Cochrane Database Syst Rev* 2000; **2**: CD000067.
8. Timmer A, McDonald JWD, Tsoulis DJ, *et al.* Azathioprine and 6-mercaptopurine for maintenance of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2012; **9**: CD000478.
9. Fraser AG, Orchard TR, Jewell DP. The efficacy of azathioprine for the treatment of inflammatory bowel disease: a 30 year review. *Gut* 2002; **50**: 485–9.
10. Chaparro M, Ordás I, Cabré E, *et al.* Safety of thiopurine therapy in inflammatory bowel disease: long-term follow-up study of 3931 patients. *Inflamm Bowel Dis* 2013; **19**: 1404–10.
11. Connell WR, Kamm MA, Ritchie JK, *et al.* Bone marrow toxicity caused by azathioprine in inflammatory bowel disease: 27 years of experience. *Gut* 1993; **34**: 1081–5.
12. Anstey A, Lennard L, Mayou SC, *et al.* Pancytopenia related to azathioprine–an enzyme deficiency caused by a common genetic polymorphism: a review. *J R Soc Med* 1992; **85**: 752–6.
13. Gisbert JP, González-Lama Y, Maté J. Thiopurine-induced liver injury in patients with inflammatory bowel disease: a systematic review. *Am J Gastroenterol* 2007; **102**: 1518–27.
14. Colombel JF, Ferrari N, Debuysere H, *et al.* Genotypic analysis of thiopurine S-methyltransferase in patients with Crohn's disease and severe myelosuppression during azathioprine therapy. *Gastroenterology* 2000; **118**: 1025–30.
15. Peyrin-Biroulet L, Khosrotehrani K, Carrat F, *et al.* Increased risk for nonmelanoma skin cancers in patients who receive thiopurines for inflammatory bowel disease. *Gastroenterology* 2011; **141**: 1621–8; e1–5.
16. Beaugerie L, Brousse N, Bouvier AM, *et al.* Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. *Lancet* 2009; **374**: 1617–25.
17. Bouhnik Y, Lémann M, Mary JY, *et al.* Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. *Lancet* 1996; **347**: 215–9.
18. Treton X, Bouhnik Y, Mary J-Y, *et al.* Azathioprine withdrawal in patients with Crohn's disease maintained on prolonged remission: a high risk of relapse. *Clin Gastroenterol Hepatol* 2009; **7**: 80–5.
19. Lémann M, Mary J-Y, Colombel J-F, *et al.* A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology* 2005; **128**: 1812–8.
20. Kim PS, Zlatanic J, Korelitz BI, *et al.* Optimum duration of treatment with 6-mercaptopurine for Crohn's disease. *Am J Gastroenterol* 1999; **94**: 3254–7.
21. Vilien M, Dahlerup JF, Munck LK, *et al.* Randomized controlled azathioprine withdrawal after more than two years treatment in Crohn's disease: increased relapse rate the following year. *Aliment Pharmacol Ther* 2004; **19**: 1147–52.
22. Lobel EZ, Korelitz BI, Xuereb MA, *et al.* A search for the optimal duration of treatment with 6-mercaptopurine for ulcerative colitis. *Am J Gastroenterol* 2004; **99**: 462–5.
23. Cassinotti A, Actis GC, Duca P, *et al.* Maintenance treatment with azathioprine in ulcerative colitis: outcome and predictive factors after drug withdrawal. *Am J Gastroenterol* 2009; **104**: 2760–7.
24. Hawthorne AB, Logan RF, Hawkey CJ, *et al.* Randomised controlled trial of azathioprine withdrawal in ulcerative colitis. *BMJ* 1992; **305**: 20–2.
25. French H, Mark Dalzell A, Srinivasan R, *et al.* Relapse rate following azathioprine withdrawal in maintaining remission for Crohn's disease: a meta-analysis. *Dig Dis Sci* 2011; **56**: 1929–36.
26. Louis E, Mary J-Y, Vernier-Massouille G, *et al.* Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab

therapy is stopped. *Gastroenterology* 2012; **142**: 63–70 e5; quiz e31.
27. Cottone M, Kohn A, Daperno M, *et al.* Advanced age is an independent risk factor for severe infections and mortality in patients given anti-tumor necrosis factor therapy for inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2011; **9**: 30–5.
28. Matsumoto S, Miyatani H, Yoshida Y. Ulcerative colitis: comparison between elderly and young adult patients and between elderly patients with late-onset and long-standing disease. *Dig Dis Sci* 2013; **58**: 1306–12.
29. Ha CY, Katz S. Clinical outcomes and management of inflammatory bowel disease in the older patient. *Curr Gastroenterol Rep* 2013; **15**: 310.
30. D'Haens G, Ferrante M, Vermeire S, *et al.* Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 2218–24.
31. Sokol H, Seksik P, Nion-Larmurier I, *et al.* Current smoking, not duration of remission, delays Crohn's disease relapse following azathioprine withdrawal. *Inflamm Bowel Dis* 2010; **16**: 362–3.

8.3 Discussion of paper

In this paper, I demonstrated relapse rates following withdrawal of thiopurines for treatment of Crohn's disease of 24% at 1 year and 39% at 2 years. Elevated inflammatory markers at the time of stopping thiopurine were associated with an increased risk of relapse. Although retreatment was usually successful, a small proportion of patients had a severe enough relapse to warrant hospitalisation or even surgery. This study also provided a useful foundation on which to develop my subsequent project on withdrawal of anti-TNF detailed in the next chapter.

9 Relapse after withdrawal from anti-TNF therapy for inflammatory bowel disease: an observational study, plus systematic review and meta-analysis

9.1 Introduction to paper

As discussed in the introduction to chapter 8, physicians and patients need to consider withdrawal of treatments in patients with Crohn's disease who are in stable remission. For biologic therapies including anti-TNF, there is stronger pressure to do so informed by the NICE recommendations. In the NICE technology appraisal for infliximab and adalimumab in Crohn's disease[87], the National Institute of Health and Care Excellence recommend that patients should be reviewed at least every 12 months to determine whether ongoing treatment is clinically appropriate. This is at least in part informed by the high cost of biologic therapy to the National Health Service.

In this study, I co-ordinated collection of data from 20 UK sites to determine the relapse rate following withdrawal of these drugs and identify factors predictive of relapse. Despite the NICE recommendations, it was difficult to identify many patients who had been withdrawn for sustained clinical remission; in Edinburgh, I was only able to find nine patients meeting the criteria from over 200 searched. Overall, I reported results on 146 Crohn's disease patients from 21 centres around the UK.

9.2 Contributions

I designed the study alongside Charlie Lees. I collected the Edinburgh data and co-ordinated data collection from the other 20 sites. I analysed the data. I then wrote the paper working with Charlie Lees and Catriona Basquill.

Published as: Chapter 7.Kennedy NA, Warner B, Johnston EL, Flanders L, Hendy P, Ding NS, et al. Relapse after withdrawal from anti-TNF therapy for inflammatory bowel disease: an observational study, plus systematic review and meta-analysis. Aliment Pharmacol Ther 2016;43:910–23. Available from: https://dx.doi.org/10.1111/apt.13547.

AP&T Alimentary Pharmacology and Therapeutics

Relapse after withdrawal from anti-TNF therapy for inflammatory bowel disease: an observational study, plus systematic review and meta-analysis

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[1]Other members of the anti-TNF withdrawal study group can be found in Appendix 1.

Publication data
Submitted 23 July 2015
First decision 11 August 2015
Resubmitted 7 December 2015
Resubmitted 11 January 2016
Resubmitted 12 January 2016
Accepted 14 January 2016
EV Pub Online 19 February 2016

As part of AP&T's peer-review process, a technical check of this meta-analysis was performed by Y. Yuan. This article was accepted for publication after full peer-review.

SUMMARY

Background
Infliximab and adalimumab have established roles in inflammatory bowel disease (IBD) therapy. UK regulators mandate reassessment after 12 months' anti-TNF therapy for IBD, with consideration of treatment withdrawal. There is a need for more data to establish the relapse rates following treatment cessation.

Aim
To establish outcomes following anti-TNF withdrawal for sustained remission using new data from a large UK cohort, and assimilation of all available literature for systematic review and meta-analysis.

Methods
A retrospective observational study was performed on 166 patients with IBD (146 with Crohn's disease (CD) and 20 with ulcerative colitis [UC) and IBD unclassified (IBDU)] withdrawn from anti-TNF for sustained remission. Meta-analysis was undertaken of all published studies incorporating 11 further cohorts totalling 746 patients (624 CD, 122 UC).

Results
Relapse rates in the UK cohort were 36% by 1 year and 56% by 2 years for CD, and 42% by 1 year and 47% by 2 years for UC/IBDU. Increased relapse risk in CD was associated with age at diagnosis [hazard ratio (HR) 2.78 for age <22 years], white cell count (HR 3.22 for >5.25 $\times$ 10^9/L) and faecal calprotectin (HR 2.95 for >50 μg/g) at drug withdrawal. Neither continued immunomodulators nor endoscopic remission were predictors. In the meta-analysis, estimated 1-year relapse rates were 39% and 35% for CD and UC/IBDU respectively. Retreatment with anti-TNF was successful in 88% for CD and 76% UC/IBDU.

Conclusions
Assimilation of all available data reveals remarkable homogeneity. Approximately one-third of patients with IBD flare within 12 months of withdrawal of anti-TNF therapy for sustained remission.

Aliment Pharmacol Ther 2016; **43**: 910–923

doi:10.1111/apt.13547

INTRODUCTION

Tumour necrosis factor (TNF) antagonists, notably infliximab (IFX) and adalimumab (ADA) are firmly established induction and maintenance agents in Crohn's disease (CD) and ulcerative colitis (UC).[1–4] The European Crohn's and Colitis Organisation (ECCO) recommend their use for CD that is refractory to steroids or relapses after initial therapy, as second-line therapy in patients with acute severe UC and in patients with immunomodulator-refractory UC.[5, 6] However, despite the advent of biosimilar infliximab, the drugs are expensive (approximately £6–10 000 per annum)[7] and there remain some concerns over long-term safety. Serious potential adverse effects include immunogenicity, opportunistic infections, melanoma.[8, 9] Once sustained deep remission has been achieved on maintenance anti-TNF therapy clinicians, patients and payers may all have different motivations for a trial of drug withdrawal. Indeed in the UK, the National Institute for Clinical Excellence (NICE) and the Scottish Medicines Consortium (SMC) mandate reassessment at 12 monthly intervals with a consideration of drug cessation where patients are in stable remission. However, there is presently insufficient data on relapse and recapture rates to inform such decision making.[9–12] We therefore aimed to examine the rate of disease relapse in IBD patients utilising all available data. We recruited a large retrospective uncontrolled cohort of patients from the UK, all withdrawn from anti-TNF therapy for sustained clinical remission, and assessed possible predictive factors for relapse and the success of drug reintroduction. We then performed a systematic review of the published literature and conference abstracts with a meta-analysis of all relevant data.

SUBJECTS AND METHODS

Study design

A multi-centre retrospective clinical audit was conducted using patients identified from 21 IBD centres across the UK. A detailed review of case notes was performed using a standardised proforma and study guide, accessible through the www.ibdscotland.org website. Data were extracted detailing patient demographics including: sex, diagnosis (CD/UC/IBDU), date of and age at diagnosis, weight (at withdrawal) and smoking status. Drug therapy details gathered include: anti-TNF used, start date, age when started, original approach of therapy, initial and maintenance dosages, stop date, age at withdrawal, tapering at withdrawal and concomitant medication. Parameters at withdrawal included: reason for withdrawal, date of last symptomatic flare and course of systemic corticosteroids prior to withdrawal, Montreal classification and behaviour, laboratory markers [faecal calprotectin, C-reactive protein (CRP), haemoglobin, platelets, erythrocyte sedimentation rate (ESR), white cell count (WCC), albumin], endoscopic findings and abdominal imaging. Endoscopic findings were given as free text by the individual sites and coded centrally by a single researcher as quiescent mild, moderate or severe. Formal assessment of the endoscopic appearances using a validated score was not deemed feasible. Relapse was also recorded, noting the severity, anti-TNF reintroduction and need for additional treatment. Eligible patients were identified for the study by searching IBD databases and out-patient clinic lists at the participating centres.

Patients with IBDU and UC were analysed as a single group since numbers of each individually were small.

Study criteria

Inclusion criteria were: confirmed diagnosis of IBD, at least 12 months of continuous anti-TNF therapy, withdrawal for sustained clinical remission and corticosteroid-free remission for at least 6 months at time of withdrawal. Patients meeting inclusion criteria were identified at each study site, and their suitability for inclusion was checked centrally based on the reported reasons for drug withdrawal and timing of last symptomatic flare, drug withdrawal and follow-up. Each study site was asked to identify patients by screening all of their patients treated at any time with anti-TNF in order to reduce bias.

Disease relapse was classified as either moderate or severe. Moderate relapse was defined by the requirement of oral steroids, immunomodulators or recommencement of anti-TNF therapy. Hospital admission, IV steroids and resectional surgery defined severe relapse.

The pre-specified primary end-point was a moderate–severe relapse at 12 months while secondary end-point was moderate–severe relapse at 24 months.

Statistical analysis

Data were collected by each site in a Microsoft Excel spreadsheet (Microsoft Corp, Redmond, WA, USA) and submitted to the lead site. Anonymised data were then collated in a single master spreadsheet. Each entry was rechecked to make sure they met the inclusion criteria. Data were analysed using R 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria). Survival analysis including Cox proportional hazards and Kaplan–Meier analysis were done using the *survival* package.[13, 14] The overall moderate-to-severe relapse rates were estimated

using the Kaplan–Meier method. These were divided into moderate and severe relapse based on the proportions of relapses of each category by that time point.

For Cox proportional hazards analysis, continuous variables were analysed using untransformed values for those with approximately normal distributions, and log-transformed values for those with approximately log-normal distributions (CRP and faecal calprotectin). For univariable analysis of variables with missing data, only individuals with known data were included. Colonoscopies were categorised as quiescent, mild and moderate inflammation, and for statistical analysis were split into no inflammation vs. a mild or greater degree of inflammation. Blood tests were only analysed for those patients without an additional reason for anti-TNF withdrawal which might have influenced the results. Continuous variables that were significant on univariable analysis were also analysed as a categorical variable using a threshold derived that gave the highest sum of sensitivity and specificity for predicting relapse at 12 months. Multivariable analysis was performed on variables with $P < 0.1$ on univariable analysis and with at least 100 individuals with data. After creating an initial model, backwards step-wise regression was performed using the Akaike An Information Criterion (AIC) to select which variables to keep. A second model was created which also included faecal calprotectin, since it was one of the most significant and clinically relevant markers; this could only include the subset of patients with faecal calprotectin results.

Systematic review

Criteria for including studies. Types of studies: Retrospective or prospective uncontrolled or controlled studies.

Types of participants: Patients with IBD withdrawn from anti-TNF therapy after a period of sustained clinical remission (at least 6 months).

Types of interventions: Withdrawal of anti-TNF therapy.

Types of outcome measures: Proportion of patients experiencing clinical relapse by 1 year following treatment withdrawal.

Exclusion criteria: Studies without an estimate of 1-year relapse; studies where the outcome measure was endoscopic recurrence rather than clinical relapse; studies where anti-TNF was being used as post-operative prevention of recurrence.

Search methods for identification of studies. Computer-assisted searches of PubMed and EMBASE were carried out covering the years 1950–2015 (PubMed) and 1980–2015 (EMBASE). PubMed search terms used were: Search (anti-TNFa OR antiTNF OR antiTNFa OR "anti-tumour necrosis" OR "anti-tumor necrosis" OR infliximab OR adalimumab OR anti-TNF OR golimumab OR certolizumab) AND (withdrawal OR discontinuation OR cessation OR stopping OR de-escalation) AND (inflammatory bowel disease OR IBD OR Crohn's OR colitis OR Crohn). EMBASE was searched using the same strategy, but combining three searches together with the AND operator. No limits were imposed on either type of search, and searches were last updated on 6 March 2015. Where available, EMBASE search results were assigned PubMed IDs using the PubMed batch citation tool. The results of all searches were then merged with those from PubMed and duplicates removed by matching PubMed IDs and manual matching of titles/journals. Where an identical abstract had been presented at two or more conferences, these were also regarded as duplicates. EMBASE includes conferences from 2009 onwards.

Data collection and analysis: All titles identified by the above searches were reviewed. Abstracts and full texts of relevant papers that related to withdrawal of anti-TNF in IBD were reviewed to identify independent data sets that met the inclusion criteria. Data were extracted and stored in an Excel spreadsheet by a single researcher (NAK). Studies were assessed as to whether they were prospective or retrospective, controlled or uncontrolled. Data extracted included an estimate of the 12-month relapse rate (controlling for loss-to-follow-up where relevant) and variables predictive of relapse.

Meta-analysis

Meta-analysis was performed using the metafor package in R 3.2.2.[15] Proportions were used as the measure of effect size and a random effects model to estimate the average proportion. Weighting was done with the inverse variance method. Proportion data were transformed using the arcsine square root transformation and reverse transformed for display. 0.5 was added to each count where there was a zero (e.g. in the situation of 100% success with retreatment). Heterogeneity was estimated using the restricted maximum-likelihood estimator. An I^2 of less than 40% was regarded as likely to be unimportant.[16] A P value for heterogeneity was also calculated using Cochran's Q method.[17] Publication bias was assessed using a funnel plot. The primary analysis was performed

using studies that included patients with at least 12 months' therapy. A further analysis was performed also including studies with a shorter minimum time on anti-TNF, though still only examining studies where patients were withdrawn from maintenance therapy.

RESULTS

Retrospective UK cohort

Out of the 21 centres across the UK, 166 patients, 146 with CD and 20 with UC/IBDU, were eligible for inclusion (Table 1). A further 19 patients were submitted but excluded from analysis, most commonly for less than 12 months' therapy or missing data. The number of screened patients was not available across most sites, but for Edinburgh 380 patients were screened to identify 10 that met the inclusion criteria with the majority of the remainder either continuing with anti-TNF (n = 147) or having been withdrawn for reasons other than sustained remission (n = 155).

One hundred and seventeen (80%) CD patients and 19 (95%) UC/IBDU patients were on infliximab prior to withdrawal; the remainder were on adalimumab. The median time taken for introducing anti-TNF therapy post-

Table 1 | Demographics of patients in the UK retrospective study

	Crohn's disease (n = 146)	Ulcerative colitis/IBDU (n = 20)
Anti-TNF used		
Infliximab	117 (80%)	19 (95%)
Adalimumab	29 (20%)	1 (5%)
Sex		
Female	83 (57%)	8 (40%)
Age at anti-TNF withdrawal/years	31 (24–42)	40 (29–46)
Reason for starting anti-TNF		
Failure of immunomodulators	117/139 (84%)	14/18 (78%)
Early combination therapy	7/139 (5%)	0
Early monotherapy	3/139 (2%)	0
Hospitalisation for acute severe disease	5/139 (4%)	4/18 (22%)
Other	7/139 (5%)	0
Time on anti-TNF/months	29 (18–45)	21 (14–33)
Follow-up time since withdrawal/months	24 (15–38)	23 (15–35)
Year stopped anti-TNF	2012 (2010–2012)	2012 (2011–2013)
Smoking at withdrawal		
Current	14/129 (11%)	1/17 (6%)
Ex	18/129 (14%)	3/17(18%)
Never	97/129 (75%)	13/17 (76%)
Montreal location		
L1 ± L4	18/142 (13%)	
L2 ± L4	38/142 (27%)	
L3 ± L4	81/142 (57%)	
L4	5/142 (4%)	
Montreal behaviour		
B1	98/142 (69%)	
B2	21/142 (15%)	
B3	23/142 (16%)	
Montreal extent		
E2		9/19 (47%)
E3		10/19 (53%)
Previous surgical resection for IBD	35/125 (28%)	0/17 (0%)
Therapy at withdrawal		
Azathioprine	66/146 (45%)	12/20 (60%)
Mercaptopurine	9/145 (6%)	1/20 (5%)
Methotrexate	20/145 (14%)	2/20 (10%)
Mesalazine	17/146 (12%)	7/20 (35%)
Any of the above	107/145 (73%)	16/20 (80%)

Numbers shown are medians and interquartile ranges or numbers and percentages as appropriate. Percentages have been calculated after exclusion of missing data within each category.

diagnosis was 63 months for CD [interquartile range (IQR) 30–122] and 22 months for UC/IBDU (IQR 10–70). Median therapy duration prior to withdrawal was 29 months (IQR 18–45) for CD and 21 months for UC/IBDU (IQR 14–33). Median follow-up was 24 months (IQR 15–38) for CD and 23 months (IQR 15–35) for UC/IBDU. Investigations at withdrawal are shown in Table 2.

The majority of patients in both disease groups (80% CD, 78% UC/IBDU) commenced anti-TNF following failure of immunomodulators. Among the CD cohort, 69% had inflammatory (Montreal B1) disease, with the remainder split between stricturing (B2) and penetrating (B3). While all patients had to be in clinical remission for 6 months at the point of treatment withdrawal, there was an additional factor that influenced the decision for withdrawal in 21 (14%) CD patients and 1 (5%) UC/IBDU patient, including planned pregnancy or mild drug intolerance (Table S1).

Relapse rate and predictive factors

By time of last follow-up, 75/146 (51%) CD patients and 9/20 (45%) UC/IBDU patients had experienced relapse (Figure 1). By 12 months, the estimated moderate-to-severe relapse rate was 36% in CD [95% confidence interval (CI) 29–44] and 42% in UC/IBDU (95% CI 15–60) at 12 months. By 24 months, the estimated relapse rates had increased to 56% in CD (95% CI 46–64) and 47.1% in UC/IBDU (95% CI 19–65). There was no significant difference in relapse rates between CD and UC/IBDU ($P = 0.95$).

Predictive factors assessed for relapse are shown in Table 3. Relapse in CD was associated with younger age at diagnosis ($P = 0.007$), white cell count at time of anti-TNF withdrawal ($P = 0.013$), isolated L4 disease ($P = 0.005$), absence of perianal disease ($P = 0.045$), Montreal behaviour B2 ($P = 0.024$) and log (faecal calprotectin) ($P = 0.041$). Stratifying patients into groups based on faecal calprotectin with a cut-off of 50 μg/g showed clear separation of the survival curves, with $P = 0.006$ (Figure 2). On multivariable analysis of variables with univariable $P < 0.1$ and $n > 100$ (Table 3b), age at diagnosis ($P = 0.002$) and white cell count $>5.25 \times 10^9$ ($P = 0.022$) remained significant. This analysis included the 128 patients with data for all included variables faecal calprotectin >50 μg/g was also significant when included in a multivariable model ($P = 0.016$), though this reduced the number of assessable patients to 42. A score comprised of white cell count, age at diagnosis and faecal calprotectin using the thresholds described above showed significant separation of survival curves ($P < 0.001$, Figure 3).

There were no associations with any predictive factors for UC/IBDU.

Consequences of relapse and re-treatment

Among the 48 CD patients who relapsed in the first 12 months, 22 (46%) required systemic corticosteroid therapy, 7 (15%) required hospital admission and 1 (2%) underwent surgery. Among UC/IBDU patients relapsing in the first 12 months, four (50%) required systemic corticosteroids and 1 (12%) underwent colectomy.

Reintroduction of anti-TNF therapy

Anti-TNF therapy was reintroduced in 56/75 (75%) CD patients and 3/9 (33%) UC/IBDU patients with relapse.

Table 2 | Investigations at withdrawal of anti-TNF in the UK retrospective study

	Crohn's disease		Ulcerative colitis/IBDU	
	n	Median (IQR) or *n* (%)	*n*	Median (IQR) or *n* (%)
Haemoglobin (g/L)	133	137 (128–146)	20	132 (126–142)
White cell count (10^9/L)	133	6.2 (5.0–7.4)	20	6.6 (5.4–8.0)
Platelet count (10^9/L)	133	256 (213–299)	20	260 (216–351)
Albumin (g/L)	128	44 (40–46)	19	39 (37–44)
CRP (mg/L)	129	2.5 (1.5–3.0)	18	2.2 (1.5–4.5)
Faecal calprotectin (μg/g)	46	46 (20–91)	3	<20 (<20–334)
Colonoscopy				
Quiescent	84	74 (88%)	16	12 (75%)
Mild		9 (11%)		2 (12%)
Moderate		1 (1%)		2 (12%)

For all blood tests, patients were only included in this analysis if they had no additional reasons for anti-TNF withdrawal ($n = 138$ for Crohn's disease and 20 for ulcerative colitis/IBDU). No full blood count was performed at withdrawal on five CD patients (three of whom were children). Colonoscopy was performed on 84 of the Crohn's disease patients and 16 of the ulcerative colits/IBDU patients.

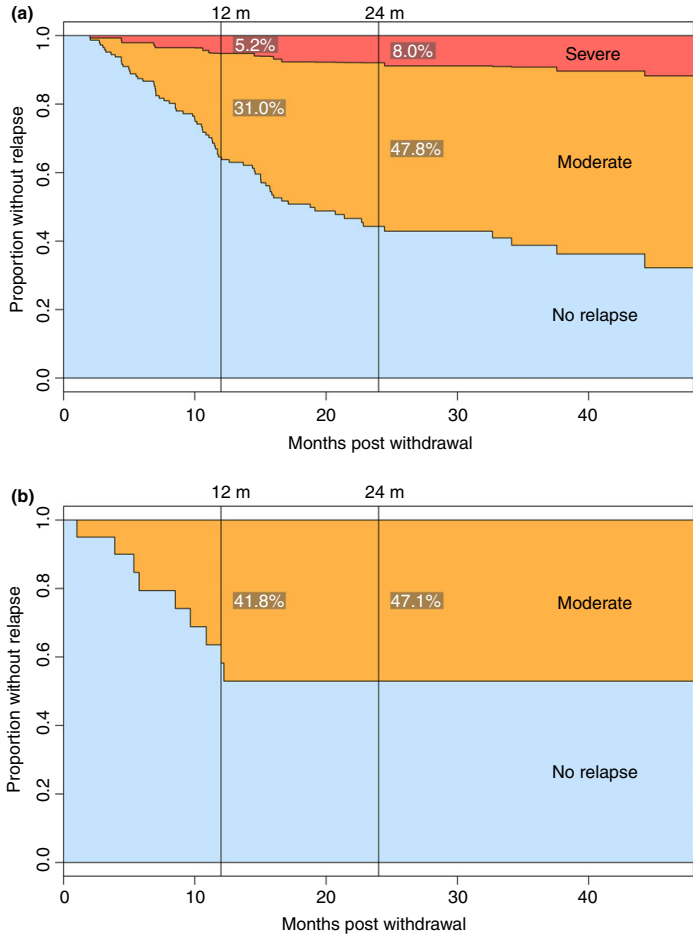

Figure 1 | Survival analysis of relapse following withdrawal of anti-TNF for sustained remission of Crohn's disease (a) and ulcerative colitis/IBD unclassified (b) in the UK retrospective study.

The same anti-TNF was reintroduced in 47/56 (84%) CD patients and 3/3 (100%) UC/IBDU patients, with the remainder switching from infliximab to adalimumab. Reintroduction was deemed successful in 52/56 (93%) with CD and 2/3 (67%) with UC/IBDU. However, in 21 of these 52 CD patients (40%) systemic steroids were also required, and in 2/52 (4%) resectional surgery was needed. None of the three UC/IBDU patients in whom anti-TNF was introduced required surgery, though the unsuccessful UC/IBDU patient required systemic steroids.

Systematic review

Initial searches and review of bibliographies identified 2629 papers after removal of duplicates (Figure 4). Sixteen studies were deemed eligible for inclusion in the meta-analysis in addition to the present study. Overall, 12 studies covered CD only, 1 UC only and 4 both diseases (Table S2, excluded studies in Table S3). All of the included studies were uncontrolled observational studies, with a mixture of prospective and retrospective approaches. Twelve studies met the inclusion criteria for the primary meta-analysis (at least 12 months' anti-TNF therapy prior to withdrawal).

Meta-analysis

In the primary meta-analyses, 624 CD patients from 10 studies and 122 UC/IBDU patients from 4 studies were included. The estimated average 12-month relapse rate

Table 3 | Predictive factors for relapse after withdrawal from anti-TNF in Crohn's disease using Cox proportional hazards model in the UK retrospective study. (a) univariable analysis; (b) multivariable analysis

(a)

	n	HR (95% CI)	*P*
Sex			
Male	146	1.22 (0.77–1.93)	0.389
Smoking at withdrawal			
Never	129	Reference	
Current		1.29 (0.65–2.56)	0.459
Ex		0.72 (0.32–1.59)	0.416
Age at diagnosis (years)	**144**	**0.97 (0.94–0.99)**	**0.007**
Age at diagnosis < 22 years	**144**	**2.71 (1.66–4.43)**	**<0.0001**
Age when starting anti-TNF (years)	**145**	**0.98 (0.96–1.00)**	**0.046**
Additional reason for anti-TNF withdrawal	146	0.66 (0.32–1.38)	0.270
Tapered at withdrawal	145	1.02 (0.37–2.79)	0.975
Montreal location			
L1	142	Reference	
L2		1.82 (0.72–4.58)	0.203
L3		2.05 (0.87–4.84)	0.100
L4		**5.43 (1.65–17.93)**	**0.005**
Montreal behaviour			
B1	142	Reference	
B2		**1.93 (1.09–3.40)**	**0.024**
B3		0.52 (0.24–1.09)	0.084
Perianal disease	**142**	**0.54 (0.30–0.99)**	**0.045**
Immunomodulator at withdrawal	146	0.68 (0.43–1.08)	0.101
Immunomodulator or 5ASA at withdrawal	146	0.77 (0.47–1.28)	0.316
Previous surgical resection	125	1.44 (0.86–2.39)	0.163
Haemoglobin (g/L)	133	1.01 (0.99–1.04)	0.147
White cell count (10^9/L)	**133**	**1.16 (1.03–1.30)**	**0.013**
White cell count >5.25 × 10^9/L	**133**	**2.54 (1.39–4.66)**	**0.003**
Platelet count (10^9/L)	133	1.00 (1.00–1.01)	0.326
CRP [$\log_{10}$ (mg/L)]	129	0.83 (0.44–1.55)	0.557
Albumin (g/L)	128	1.00 (0.94–1.05)	0.891
Faecal calprotectin >50 μg/g	**46**	**3.32 (1.42–7.79)**	**0.006**
Faecal calprotectin [$\log_{10}$ (μg/g)]	**46**	**1.82 (1.03–2.82)**	**0.041**
Inflammation at colonoscopy	84	0.93 (0.39–2.20)	0.863

(b)

	Model without calprotectin (n = 128)		Model with calprotectin (n = 42)	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age at diagnosis <22 years	**2.29 (1.35–3.88)**	**0.002**	**2.78 (1.11–7.00)**	**0.03**
Montreal behaviour				
B1	Reference			
B2	1.60 (0.88–2.90)	0.200		
B3	0.51 (0.24–1.09)	0.089		
White cell count >5.25 × 10^9/L	**2.06 (1.11–3.80)**	**0.022**	**3.22 (0.95–10.93)**	**0.06**
Faecal calprotectin >50 μg/g			**2.95 (1.22–7.12)**	**0.02**

HR, hazard ratio; CI, confidence interval.

P values less than 0.05 are highlighted in bold. For continuous variables, hazard ratios shown are for each unit increase for age, haemoglobin, white cell count, platelet count and albumin. For CRP and calprotectin which have a log-normal distribution, hazard ratios shown are for each 10-fold increase.

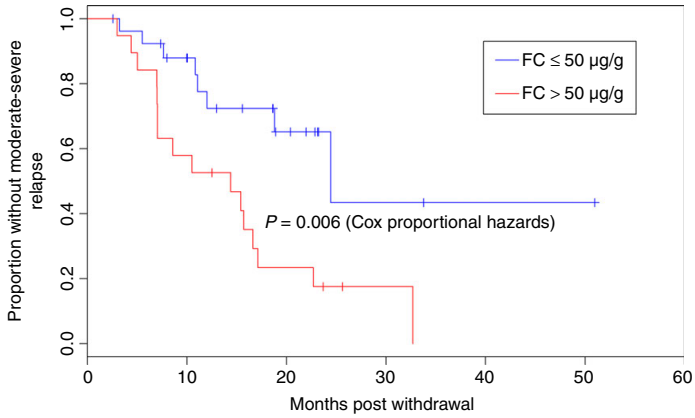

Figure 2 | Relapse in Crohn's disease patients following withdrawal of anti-TNF stratified by faecal calprotectin (FC) (n = 46) in the UK retrospective study.

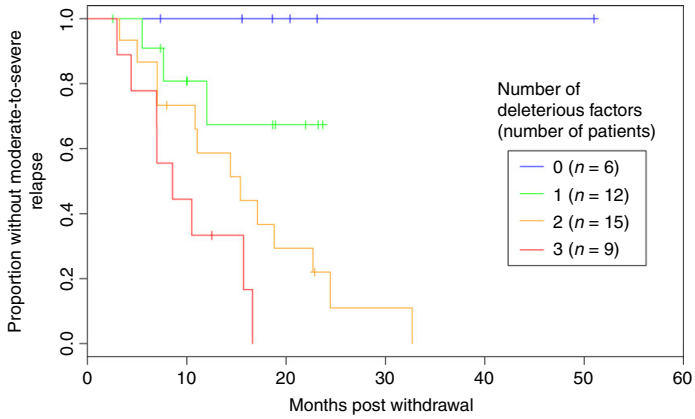

Figure 3 | Relapse in Crohn's disease patients following withdrawal of anti-TNF stratified by faecal calprotectin, white cell count and age at diagnosis in the UK retrospective study.

was 39% for CD (95% CI 35–44) and 35% for UC/IBDU (95% CI 26–43) (Figure 5). Both data sets had low heterogeneity, with I^2 = 12% for CD and 0% for UC/IBDU. Expanding the inclusion criteria to include patients with shorter periods on anti-TNF prior to drug withdrawal increased the heterogeneity to I^2 = 40% for CD and 56% for UC (Figure S1).

For CD, the estimated average 24-month relapse rate was 54% (95% CI 49–59) when using the four studies with relevant data that met the primary inclusion criteria (Figure S2A). Extending this to all eight studies with available data gave a similar estimated average relapse rate of 53% (95% CI 49–57) (Figure S3). For UC, there were only two studies with 24-month relapse date. The estimated average relapse rate was 42% (95% CI 27–58) (Figure S2B).

The estimated average rate of success of retreatment was 88% for CD (95% CI 78–95) and 76% for UC/IBDU (95% CI 56–92) (Figure S4). For CD, there was significant heterogeneity of retreatment success (I^2 = 73%, P < 0.01), though this disappeared when the Monterubbianesi[18] study was removed (I^2 without Monterub-

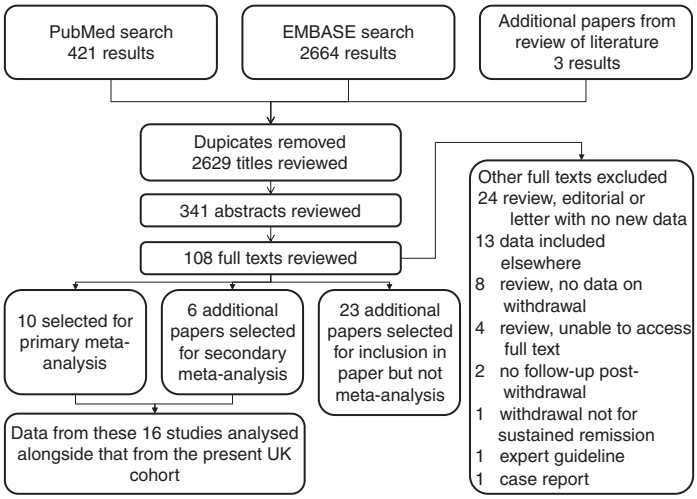

Figure 4 | Inclusion flowchart for systematic review/meta-analysis.

bianesi study 38%, $P = 0.21$). For UC, the total numbers were low (28 individuals across three studies) and so the confidence intervals were wide.

A funnel plot to assess for publication bias was symmetric for CD (Figure S5), though there was a single outlier (the paediatric Wynands *et al.* study[19]). There were too few points to make a meaningful assessment of publication bias for UC/IBDU.

DISCUSSION

In patients with IBD withdrawn from anti-TNF therapy while in sustained remission, our meta-analysis has shown a clinical relapse rate by 12 months of 39% for CD and 37% for UC/IBDU. For CD, the 24-month relapse rate was 54%. These estimates are based on our large UK retrospective uncontrolled cohort and assimilation of all presently available and relevant data from the literature. The meta-analysis reported herein is remarkable for the lack of heterogeneity among the individual data sets. With the important caveat that relatively fewer UC patients were available for analysis, the relapse rates are broadly similar to those observed in CD.

These data will give confidence to clinicians when discussing with patients established on anti-TNF therapy the chances of disease flare if the drugs are withdrawn. Approximately one in three patients with any form of inflammatory bowel disease are likely to experience a moderate-to-severe flare within 12 months of drug withdrawal, and one in two by 24 months. These odds are likely to seem unfavourable to many clinicians and patients but should prove useful when set alongside other key factors. These might include how the timing of drug withdrawal fits in with a patient's life (e.g. important education, work, or family events). In some countries, such as parts of the UK, drug withdrawal will be recommended by regulatory authorities.

Two other key pieces of information are likely to be useful to fully inform clinical teams about making key alterations in drug therapy. Firstly, if a patient does experience a disease flare following drug withdrawal, what are the consequences of this and how successful is reintroduction of drug therapy? Our large UK cohort offers some useful guidance on this. While just under one half of all patients who relapsed required systemic corticosteroids, hospitalisation rates were relatively low (17% in CD, zero in UC) and surgical rescue was a rare event (only two patients). When anti-TNF therapy was restarted it was deemed to be successful in over 90% of patients with CD. These results were confirmed in the meta-analysis, with an estimated retreatment success rate of 88% (95% CI 78–95). There was a single outlying study (Monterubbianesi[18]); the reasons for this are unclear, particularly since this study has only been published as a conference abstract, but may reflect different criteria for retreatment success. We have insufficient data to draw any meaningful conclusions in UC.

Secondly, what are the predictive factors of relapse at the time of drug withdrawal? Arguably this is the most

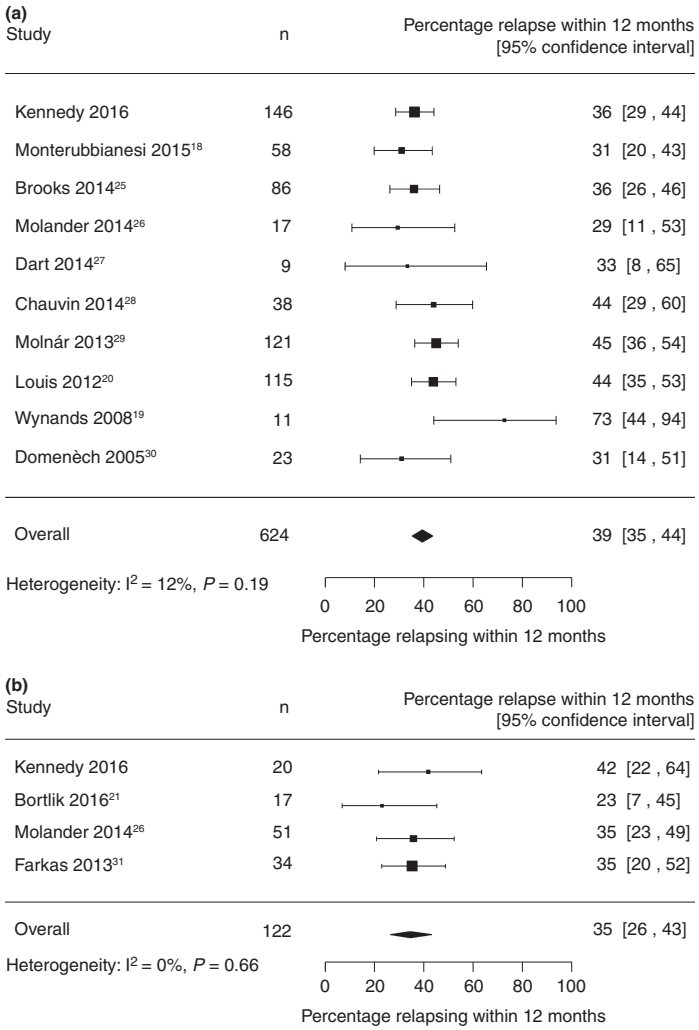

Figure 5 | Forest plot for relapse by 12 months after anti-TNF withdrawal for CD (a) and UC/IBDU (b).

important piece of information to enable rationale stratification of patients at drug withdrawal. In our cohort on multivariable analysis, younger age at diagnosis, white cell count and faecal calprotectin were predictive of relapse at 12 months in CD. In contrast, evidence of disease activity at colonoscopy was not predictive, though only 12% of those colonoscoped had any evidence of disease activity. In fact, of the six factors identified in the simplified model of the STORI study, only faecal calprotectin and white cell count were significant in the present study.[20] L4 disease was also predictive, but the generalisability of this finding is limited by the small numbers in that disease group (five patients).

At a fundamental level we still cannot address the question of whether evidence of active disease at drug withdrawal predicts disease relapse. Central to this paradox is the question whether patients on anti-TNF therapy with complete mucosal healing are in complete remission because of their ongoing therapy or in spite of it. One might expect those patients who exhibit ongoing

mucosal inflammation despite being in complete clinical remission to be at much higher risk of disease relapse following drug withdrawal. In our study, a faecal calprotectin above 50 μg/g was predictive in the Crohn's cohort of relapse (HR 3.32, $P = 0.006$). faecal calprotectin was also found to be predictive in the STORI study,[20] while Bortlik *et al.* found no association of faecal calprotectin with relapse.[21] Inconsistencies across the literature in large part reflect that in most cohorts very few patients had any objective evidence of mucosal inflammation at drug withdrawal. The data from our study are typical: 83% of patients had a normal CRP and 88% a calprotectin <250 μg/g with 60% having calprotectin <50 μg/g. Although white cell count was an independent predictive factor in this cohort, the optimum threshold was well within the normal range at 5.25×10^9/L (and was not related to immunomodulator use). In addition, full colonoscopic assessment, where it was performed, was completely normal in 86/100. This is despite clinical remission at the time of drug withdrawal being the only major inclusion criterion in our protocol. Clinicians are evidently continuing therapy in patients where there is evidence of ongoing inflammation, often in spite of drug optimisation. For many, this will reflect a lack of alternative therapeutic options. However, there are likely to be a substantial number of patients in whom anti-TNF therapy can be discontinued in this scenario as it is having no discernible impact on the disease course. This is highlighted by results from the STORI trial where undetectable infliximab trough levels were associated with a lower risk of subsequent relapse on drug withdrawal.[20] Further prospective clinical trials are planned to address these key issues.

The limitations of our UK cohort are its retrospective nature, and the missing data at the time of drug withdrawal. CRP, calprotectin and colonoscopic assessment were available in 93%, 30% and 60% of patients. The relatively low number of abnormal colonoscopies may have limited the power of the study for this predictive marker. Details of small bowel imaging were only available in 30 patients, and the data were too heterogeneous (ultrasound, CT, MRI, barium studies) to allow meaningful analysis. All sites were asked to be thorough in their searches of anti-TNF-treated patients to reduce the risk of selection bias, but this remains a potential concern. In Edinburgh, where the lead authors are based, it should be noted that even with comprehensive review of our patient cohort, it was in practice very difficult to identify patients who met our criteria. This suggests that despite NICE guidance, relatively few patients with IBD have their anti-TNF therapy stopped for sustained remission. It is also of note that 75% of CD patients in the UK cohort were never smokers, which is lower than reported elsewhere.[22–24] This may reflect clinician concerns about relapse rates in smokers whose anti-TNF therapy is withdrawn.

We have been thorough in our systematic review and followed strict criteria and guidelines for the selection of studies for the meta-analysis. However, all of the included studies were uncontrolled and many were retrospective. It is therefore not possible to draw conclusions about what would have happened in the absence of drug withdrawal. For the retrospective cohorts in particular, there is a risk of recall bias, which could inflate or reduce estimates of relapse rate in those cohorts; however, there was no significant difference in the 12-month relapse rate between prospective and retrospective cohorts.

We are, however, able to draw several important conclusions based on a synthesis of all available data. Approximately one-third of patients with CD or UC in sustained clinical remission are likely to suffer a disease relapse within 12 months of planned drug withdrawal. We are presently unable to predict which patients are most likely to flare in this situation. We can recapture disease remission by restarting anti-TNF therapy in the majority of patients, although nearly half may also require a course of corticosteroids. Clinicians and patients should weigh up the decisions about drug withdrawal on an individual basis taking into account the preceding disease course, and the appropriate time in a patient's life for such critical therapeutic changes.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Detailed reasons for withdrawal from anti-TNF.

Table S2. Characteristics of articles included in systematic review and meta-analysis of withdrawal of anti-TNF for sustained clinical remission, sorted by year of publication. (A) Studies with at least 1 year anti-TNF prior to withdrawal. (B) Studies with maintenance therapy but <1 year anti-TNF at withdrawal. C: Studies included in secondary meta-analysis but excluded from primary meta-analysis for other reasons.

Table S3. Studies excluded from meta-analysis.

Figure S1. Forest plot for relapse by 12 months after anti-TNF withdrawal for CD (A) and UC/IBDU (B) including all studies with patients treated with maintenance anti-TNF.

Figure S2. Forest plot for relapse by 24 months after anti-TNF withdrawal for CD (A) and UC/IBDU (B) including only studies with patients treated with maintenance anti-TNF for at least 12 months.

Figure S3. Forest plot for relapse by 24 months after anti-TNF withdrawal for CD including all studies of patients treated with maintenance anti-TNF.

Figure S4. Forest plot for success rates of reintroduction of anti-TNF after withdrawal for CD (A) and UC/IBDU (B) including all studies with patients treated with maintenance anti-TNF.

Figure S5. Funnel plot for relapse by 12 months after anti-TNF withdrawal including studies with patients treated with maintenance anti-TNF for at least one year prior to withdrawal.

AUTHORSHIP

Guarantor of the article: Dr Charles W. Lees.

Author contributions: NAK and CWL contributed towards study design; NAK, BW, ELJ, LF, PH, NSD, RH, ASF, CB, CAL, FLC, DG, with supervision from CDM, MP, IG, TA, SM, JL, JG, JS, AH, SM, PMI and CWL contributed towards data collection; NAK contributed towards data analysis; CB, NAK and CWL contributed towards the writing of the paper with subsequent review and input from the other authors.

All authors approved the final version of the manuscript.

ACKNOWLEDGEMENTS

Declaration of personal interests: Nicholas Kennedy was funded by a Research Training Fellowship from the Wellcome Trust [097943]. He has received speaker fees from MSD, Takeda, Falk, Pharmacosmos and Actavis. Nik Ding has served on an advisory board and as a speaker for Abbvie and Falk. Christopher Lamb is funded by a research training fellowship from the Wellcome Trust (093885/Z/10/Z). He has served as a speaker and consultant for Genentech/Roche, a speaker for Takeda and has received research funding from Genentech/Roche. Charles Murray has served as a speaker, a consultant and an advisory board member for Abbvie, MSD, Falk and Shire. Miles Parkes has served on advisory boards for Abbvie and MSD and has received research funding from Abbvie. Dan Gaya has spoken/worked in an advisory capacity for MSD, Abbvie, Takeda, Vifor pharma and Ferring. James Lindsay has served as a speaker, a consultant and an advisory board member for Abbvie, Ferring, MSD, Takeda, Jansen, Pfizer, Actavis, Napp, Hospira, Shire and Tillott's and has received research funding from Hospira, MSD and Shire. John Gordon has served as a consultant and an advisory board member for MSD, Abbvie, Ferring and Tillott's. Jack Satsangi has received speaker fees from MSD and Takeda and research support from Abbvie. Ailsa Hart has served as a speaker, consultant and advisory board member for AbbVie, Atlantic, BMS, Janssen, MSD, Falk, Ferring, Hospira, Napp, Pfizer and Takeda, Sara McCartney has served as a speaker, a consultant and an advisory board member for MSD, Abbvie, Falk, Takeda, Actavis, Ferring and Janssen. Peter Irving has served as a speaker, a consultant and an advisory board member for Abbvie, MSD, Takeda, Genentech, Ferring, Falk, Shire, Topivert, Pharmacosmos, Tillott's, Actavis, Johnson and Johnson, Hospira and Vifor and has received research funding from MSD. Dr Charlie Lees has received research support from Abbvie and Shire, acted as a consultant to Abbvie, MSD, Hospira, Pharmacosmos, Takeda, Vifor Pharma and Dr Falk and received speaking fees and travel support from Abbvie, MSD, Takeda, Shire, Ferring, Hospira, Warner-Chilcott and Dr Falk. Ben Warner, Emma Johnston, Lucy Flanders, Phil Hendy, Richard Harris, Adam Fadra Catriona Basquill, Fiona Cameron, Ian Gooding, Steve Mann have no personal interests to declare.

Declaration of funding interests: None.

REFERENCES

1. Hanauer SB, Feagan BG, Lichtenstein GR, *et al.* Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541–9.
2. Rutgeerts P, Sandborn WJ, Feagan BG, *et al.* Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462–76.
3. Colombel JF, Sandborn WJ, Rutgeerts P, *et al.* Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52–65.
4. Schreiber S, Khaliq-Kareemi M, Lawrance IC, *et al.* Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239–50.
5. Dignass A, Van Assche G, Lindsay JO, *et al.* The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: current management. *J Crohns Colitis* 2010; **4**: 28–62.
6. Dignass A, Lindsay JO, Sturm A, *et al.* Second European evidence-based consensus on the diagnosis and management of ulcerative colitis Part 2: current management. *J Crohns Colitis* 2012; **6**: 991–1030.
7. Joint Formulary Committee. British National Formulary [Internet], 2016. Available at: http://www.medicinescomplete.com (accessed 11 January 2016).
8. Theede K, Dahlerup JF, Fallingborg J, *et al.* Biologic therapy in inflammatory bowel disease. *Dan Med J* 2013; **60**: 1–9.
9. Pariente B, Laharie D. Review article: why, when and how to de-escalate therapy in inflammatory bowel diseases. *Aliment Pharmacol Ther* 2014; **40**: 338–53.
10. Louis E, Belaiche J, Reenaers C. Anti-TNF and Crohn's disease: when should we stop? *Curr Drug Targets* 2010; **11**: 148–51.
11. Van Assche G, Lewis JD, Lichtenstein GR, *et al.* The London position statement of the world congress of gastroenterology on biological therapy for IBD with the European Crohn's and Colitis Organisation: safety. *AmJ Gastroenterol* 2011; **106**: 1594–602.
12. Sorrentino D, Nash P, Viladomiu M, Hontecillas R, Bassaganya-Riera J. Stopping Anti-TNF Agents in Patients with Crohn's Disease in Remission: is It a Feasible Long-term Strategy? *Inflamm Bowel Dis* 2014; 1–10.
13. Therneau TM. A Package for Survival Analysis in S [Internet], 2014. Available at: http://cran.r-project.org/package=survival (accessed 11 January 2016).
14. Therneau Terry M, Grambsch Patricia M. *Modeling Survival Data: Extending the Cox Model*. New York: Springer, 2000.

15. Viechtbauer W. Conducting meta-analyses in R with the metafor package. *J Stat Softw* 2010; **36**: 1–48.
16. The Cochrane Collaboration. Identifying and measuring heterogeneity, chapter 9.5.2. In: Higgins JPT, Green S, eds. *Cochrane Handbook for Systematic Reviews of Interventions*, 2011; Available from: http://handbook.cochrane.org/chapter_9/9_5_2_identifying_and_measuring_heterogeneity.htm (Accessed 11 January 2016).
17. Cochran WG. The combination of estimates from different experiments. *Biometrics* 1954; **10**: 101.
18. Monterubbianesi R, Papi C, Kohn A. P529. Maintenance of clinical remission in Crohn's disease patients after discontinuation of antiTNF agents: results from a single centre cohort. *J Crohns Colitis* 2015; **9**: S345–S345.
19. Wynands J, Belbouab R, Candon S, *et al.* 12-month follow-up after successful infliximab therapy in pediatric crohn disease. *J Pediatr Gastroenterol Nutr* 2008; **46**: 293–8.
20. Louis E, Mary J-Y, Vernier-Massouille G, *et al.* Maintenance of remission among patients with Crohn's disease on antimetabolite therapy after infliximab therapy is stopped. *Gastroenterology* 2012; **142**: 63–70. e5; quiz e31.
21. Bortlik M, Duricova D, Machkova N, *et al.* Discontinuation of anti-tumor necrosis factor therapy in inflammatory bowel disease patients: a prospective observation. *Scand J Gastroenterol* 2016; **51**: 196–202.
22. Nunes T, Etchevers MJ, Domènech E, *et al.* Smoking does influence disease behaviour and impacts the need for therapy in Crohn′s disease in the biologic era. *Aliment Pharmacol Ther* 2013; **38**: 752–60.
23. Burisch J, Pedersen N, Cukovic-Cavka S, *et al.* Environmental factors in a population-based inception cohort of inflammatory bowel disease patients in Europe — An ECCO-EpiCom study. *J Crohns Colitis* 2014; **8**: 607–16.
24. Lunney PC, Kariyawasam VC, Wang RR, *et al.* Smoking prevalence and its influence on disease course and surgery in Crohn's disease and ulcerative colitis. *Aliment Pharmacol Ther* 2015; **42**: 61–70.
25. Brooks AJ, Sebastian S, Cross SS, Robinson K, Warren L, Wright A, *et al.* Outcome of elective withdrawal of anti-tumour necrosis factor-α therapy in patients with Crohn's disease in established remission. *J Crohns Colitis* 2015. pii: jjv000. http://ecco-jcc.oxfordjournals.org/content/early/2015/05/15/j.crohns.2014.09.007.long
26. Molander P, Färkkilä M, Salminen K, Kemppainen H, Blomster T, Koskela R, *et al.* Outcome after discontinuation of TNFα-blocking therapy in patients with inflammatory bowel disease in deep remission. *Inflamm Bowel Dis* 2014; **20**: 1021–8.
27. Dart RJ, Griffin N, Taylor K, Duncan J, Sastrillo M, Sanderson J, *et al.* Reassessment of Crohn' s disease treated with at least 12 months of anti-TNF therapy: how likely is treatment withdrawal? *Frontline Gastroenterol* 2014; **5**: 176–82.
28. Chauvin A, Le Thuaut A, Belhassan M, Le Baleur Y, Mesli F, Bastuji-Garin S, *et al.* Infliximab as a bridge to remission maintained by antimetabolite therapy in Crohn's disease: a retrospective study. *Dig Liver Dis* 2014; **46**: 695–700.
29. Molnár T, Lakatos PL, Farkas K, Nagy F, Szepes Z, Miheller P, *et al.* Predictors of relapse in patients with Crohn's disease in remission after 1 year of biological therapy. *Aliment Pharmacol Ther* 2013; **37**: 225–33.
30. Domènech E, Hinojosa J, Nos P, Garcia-Planella E, Cabré E, Bernal I, *et al.* Clinical evolution of luminal and perianal Crohn's disease after inducing remission with infliximab: how long should patients be treated? *Aliment Pharmacol Ther* 2005; **22**: 1107–13.
31. Farkas K, Lakatos PL, Nagy F, Szepes Z, Miheller P, Papp M, *et al.* Predictors of relapse in patients with ulcerative colitis in remission after one-year of infliximab therapy. *Scand J Gastroenterol* 2013; **48**: 1394–8.

APPENDIX 1

FULL LIST OF CONTRIBUTORS TO 'OUTCOMES AFTER WITHDRAWAL FROM ANTI-TNF THERAPY FOR INFLAMMATORY BOWEL DISEASE: AN OBSERVATIONAL STUDY, SYSTEMATIC REVIEW AND META-ANALYSIS'

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9.3 Discussion of paper

In this paper, I demonstrated using a large multicentre cohort a risk of relapse of 36% at 1 year and 56% at 2 years following withdrawal of anti-TNF therapy in patients with Crohn's disease. These rates were highly consistent with the data in the accompanying systematic review and meta-analysis I performed. The results were also consistent across both prospective and retrospective studies, suggesting that the identification of patients for retrospective studies had been done without too much selection bias. Information on relapse rates is useful when considering withdrawal of anti-TNF, and in particular can inform discussion with a patient. Younger patients, and those with elevated white cell count or faecal calprotectin were at increased risk of relapse. Interestingly, the faecal calprotectin threshold I identified here was lower than in some other cohorts and may reflect the fact that most patients had relatively little evidence of active inflammation at baseline.

White cell count came out as a predictive factor for subsequent relapse, although in both studies the optimal threshold for predicting relapse was well within the normal range and the absolute difference in median white cell count was relatively modest. Patients included in both studies had been selected as being in clinical remission by their treating IBD team, a decision which will have been influenced by the serum CRP and faecal calprotectin where available. The use of white cell count as a marker of subclinical inflammation has been described in other disease areas,[144,145] and this is a possible explanation here. It would be of interest in future to study whether panels of inflammatory cytokines might perform better as a predictive tool.

Work done on this project, alongside the review article I co-authored at a similar point in time[146], helped inform discussions around the European Union Horizon2020-funded

BIOCYCLE program which includes a randomised-controlled trial of anti-TNF and thiopurine withdrawal (SPARE), and in which Edinburgh took the leading role in the UK. A randomised controlled trial is the optimal way to assess the effect of a specific intervention, in this case the impact of withdrawal of either a thiopurine or infliximab from combination therapy on relapse rates. If the study reaches its primary endpoint, this will provide much stronger evidence for the effect of drug withdrawal on relapse than can be inferred from cohort studies. The data from SPARE may also help understand the contribution from other clinical factors on relapse.

10 Predictors of anti-TNF treatment failure: a prospective multi-centre study of biologic naïve patients with active luminal Crohn's disease

10.1 Introduction to paper

Over the past 20 years, anti-TNF therapies have made a marked difference to the management of Crohn's disease. They have become a key tool for the induction and maintenance of remission, and now represent a significant proportion of healthcare expenditure related to Crohn's disease.[147] While they have transformed the lives of some patients, the rate of primary response is estimated to be between 10 and 40%[85,86,148], and loss of response is common.

PANTS is a prospective, UK-wide study that was set-up in 2013 to study response and treatment failure to infliximab and adalimumab. To the best of my knowledge, it represents the largest real-world study of its type in inflammatory bowel disease. 1610 patients were recruited from 120 sites and followed for up to three years. The study includes a wealth of longitudinal clinical data. Patients were seen at least four times during the first year, and twice-yearly thereafter. Patients treated with infliximab had additional visits at each 8-weekly infusion during the first year.

This large dataset has provided the opportunity to explore the relationship between baseline clinical factors, drug and anti-drug antibody concentrations and outcomes. We have also collected longitudinal biological samples, and this will permit detailed multi-omic study of drug response and non-response; more detail of planned work will be discussed in the future work section of the final discussion.

10.2 Contributions

PANTS was conceived by a team of gastroenterologists led by Tariq Ahmad. The project was managed by Claire Bewshea, and the laboratory work was co-ordinated by Tim McDonald, Mandy Perry and Rachel Nice. I began work on the PANTS data in 2016. Previous analyses had been performed by Graham Heap, but I started again with an analysis script that includes over 10,000 lines of R code and 1600 of SQL. I conducted almost all of the analyses represented in the paper below, with the exception of the predictive modelling which was done by Harry Green. I wrote the paper, primarily in conjunction with James Goodhand and Tariq Ahmad.

Published as: Chapter 8.Kennedy NA, Heap GA, Green HD, Hamilton B, Bewshea C, Walker GJ, et al. Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn's disease: a prospective, multicentre, cohort study. lancet Gastroenterol Hepatol. 2019 Feb 26 (epub ahead of print). Available from: https://dx.doi.org/10.1016/S2468-1253(19)30012-3.

Articles

Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn's disease: a prospective, multicentre, cohort study

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Summary

Background Anti-TNF drugs are effective treatments for the management of Crohn's disease but treatment failure is common. We aimed to identify clinical and pharmacokinetic factors that predict primary non-response at week 14 after starting treatment, non-remission at week 54, and adverse events leading to drug withdrawal.

Methods The personalised anti-TNF therapy in Crohn's disease study (PANTS) is a prospective observational UK-wide study. We enrolled anti-TNF-naive patients (aged ≥6 years) with active luminal Crohn's disease at the time of first exposure to infliximab or adalimumab between March 7, 2013, and July 15, 2016. Patients were evaluated for 12 months or until drug withdrawal. Demographic data, smoking status, age at diagnosis, disease duration, location, and behaviour, previous medical and drug history, and previous Crohn's disease-related surgeries were recorded at baseline. At every visit, disease activity score, weight, therapy, and adverse events were recorded; drug and total anti-drug antibody concentrations were also measured. Treatment failure endpoints were primary non-response at week 14, non-remission at week 54, and adverse events leading to drug withdrawal. We used regression analyses to identify which factors were associated with treatment failure.

Findings We enrolled 955 patients treated with infliximab (753 with originator; 202 with biosimilar) and 655 treated with adalimumab. Primary non-response occurred in 295 (23·8%, 95% CI 21·4–26·2) of 1241 patients who were assessable at week 14. Non-remission at week 54 occurred in 764 (63·1%, 60·3–65·8) of 1211 patients who were assessable, and adverse events curtailed treatment in 126 (7·8%, 6·6–9·2) of 1610 patients. In multivariable analysis, the only factor independently associated with primary non-response was low drug concentration at week 14 (infliximab: odds ratio 0·35 [95% CI 0·20–0·62], p=0·00038; adalimumab: 0·13 [0·06–0·28], p<0·0001); the optimal week 14 drug concentrations associated with remission at both week 14 and week 54 were 7 mg/L for infliximab and 12 mg/L for adalimumab. Continuing standard dosing regimens after primary non-response was rarely helpful; only 14 (12·4% [95% CI 6·9–19·9]) of 113 patients entered remission by week 54. Similarly, week 14 drug concentration was also independently associated with non-remission at week 54 (0·29 [0·16–0·52] for infliximab; 0·03 [0·01–0·12] for adalimumab; p<0·0001 for both). The proportion of patients who developed anti-drug antibodies (immunogenicity) was 62·8% (95% CI 59·0–66·3) for infliximab and 28·5% (24·0–32·7) for adalimumab. For both drugs, suboptimal week 14 drug concentrations predicted immunogenicity, and the development of anti-drug antibodies predicted subsequent low drug concentrations. Combination immunomodulator (thiopurine or methotrexate) therapy mitigated the risk of developing anti-drug antibodies (hazard ratio 0·39 [95% CI 0·32–0·46] for infliximab; 0·44 [0·31–0·64] for adalimumab; p<0·0001 for both). For infliximab, multivariable analysis of immunododulator use, and week 14 drug and anti-drug antibody concentrations showed an independent effect of immunomodulator use on week 54 non-remission (odds ratio 0·56 [95% CI 0·38–0·83], p=0·004).

Interpretation Anti-TNF treatment failure is common and is predicted by low drug concentrations, mediated in part by immunogenicity. Clinical trials are required to investigate whether personalised induction regimens and treatment-to-target dose intensification improve outcomes.

Funding Guts UK, Crohn's and Colitis UK, Cure Crohn's Colitis, AbbVie, Merck Sharp and Dohme, Napp Pharmaceuticals, Pfizer, and Celltrion.

Lancet Gastroenterol Hepatol 2019; 4: 341–53

Published Online February 26, 2019 http://dx.doi.org/10.1016/S2468-1253(19)30012-3

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See Online for appendix

Research in context

Evidence before this study

We searched PubMed from Jan 1, 1974, to Sept 18, 2018, with the terms "Crohn's disease" AND "antitumor necrosis factor" or "anti-tumour necrosis factor" or "infliximab" or "adalimumab" or "anti TNF" or "anti-TNF" or "anti-tumour necrosis factor" AND "clinical response" or "efficacy" or "primary non-response" or "immunogenicity". We cross checked the reference lists of review articles and landmark studies. We identified 11 previous systematic reviews, five with meta-analyses: 127 original articles reported factors associated with treatment failure. These studies were mostly retrospective or cross-sectional in design (n=85), done in adults (n=110), subject to tertiary centre bias (n=64), restricted to the use of infliximab (n=85), or too small to permit predictive multivariable analyses (n=75). In summary, multiple patient, disease, and pharmacokinetic factors, including anti-TNF drug and anti-drug antibody concentrations (immunogenicity), have been implicated in anti-TNF treatment failure, but their relative effects and interactions have not been fully explored, and target drug concentrations have not been validated.

Added value of this study

We enrolled 1610 patients with active luminal Crohn's disease treated with infliximab (originator and biosimilar) or adalimumab. To our knowledge, this is the largest prospective study of anti-TNF therapy in inflammatory bowel disease done so far. Patients were recruited from 120 UK hospitals, reflecting real-life practice in specialist and non-specialist inflammatory bowel disease centres. Consistent with the registration studies, about a quarter of patients had primary non-response to anti-TNF therapy, a third of initial responders lost response, and only a third were in remission at week 54. Treatment failure, safety, and proportion of patients who developed anti-drug antibodies were similar between patients taking infliximab and the biosimilar. Clinical variables that were associated with treatment failure were week 14 drug concentrations and immunogenicity. We observed a bidirectional negative relationship between drug concentration and immunogenicity: low drug concentrations at week 14 were the main factor associated with immunogenicity by week 54, and conversely immunogenicity was the main factor associated with low drug concentrations by week 54, most likely via clearance of drug. Immunogenicity was twice as common in infliximab-treated than adalimumab-treated patients at week 54, and combination therapy with a thiopurine or methotrexate mitigated this risk.

Implications of the available evidence

Anti-TNF treatment failure is associated with suboptimal drug concentrations, suggesting it might be possible to improve Crohn's disease outcomes by boosting effective drug concentrations. Dose intensification during induction in at-risk individuals (eg, patients with obesity, smokers, and patients with more active disease) and iterative dose adjustment—aiming for higher target drug concentrations than those currently recommended—might improve the durability and effectiveness of anti-TNF treatment. Reassuringly, treatment failure, safety, and immunogenicity of the infliximab biosimilar were no different to the infliximab originator; use of the biosimilar might therefore mitigate some of the cost constraints of dose intensification. Thiopurines or methotrexate should be used in all infliximab-treated patients but could be avoided in some patients treated with adalimumab.

Introduction

The anti-TNF monoclonal antibodies infliximab and adalimumab are effective treatments for patients with Crohn's disease refractory to conventional therapies. Successful treatment leads to mucosal healing, reduced hospitalisations (ie, admissions to hospital) and surgeries, and improvement in quality of life.[1–5]

Unfortunately, anti-TNF treatment failure is common: 10–40% of patients do not respond to induction therapy (primary non-response),[6–8] 24–46% of patients have secondary loss of response in the first year of treatment,[9] and approximately 10% have an adverse drug reaction that curtails treatment.[10]

Multiple patient, disease, and drug related factors have been implicated in anti-TNF treatment failure,[11] but their relative effects, interactions, and effect on drug and anti-drug antibody concentrations have not been explored in an adequately powered prospective study. Early identification of patients at risk of treatment failure might help facilitate direct monitoring, early dose optimisation, and use of strategies to mitigate the development of anti-drug antibodies, allowing these drugs to be used in a safer, more cost-effective manner.

The main aim of the personalised anti-TNF therapy in Crohn's disease study (PANTS) was to build a bio-repository to investigate the genetic and other factors associated with anti-TNF treatment failure in patients with active luminal Crohn's disease. In this Article, we report the clinical and pharmacokinetic factors associated with and predictive of anti-TNF failure in the first year of treatment.

Methods

Study design and participants

PANTS is a UK-wide, multicentre, prospective observational cohort reporting on treatment failure of the anti-TNF drugs infliximab (originator, Remicade [Merck Sharp and Dohme, Hertforshire, UK] and biosimilar, CT-P13 [Celltrion, Incheon, South Korea]) and adalimumab (Humira [AbbVie, Chicago, IL, USA]) in anti-TNF-naive patients with active luminal Crohn's disease.

Patients were recruited at the time of first anti-TNF exposure from 120 National Health Service trusts across the UK (appendix pp 3–10) between March 7, 2013, and July 15, 2016. Patients were evaluated for 12 months or until drug withdrawal.

Patients were screened for inclusion in our cohort at the time of decision to treat with an anti-TNF drug and no more than 4 weeks before starting to receive the drug. The eligibility criteria were as follows: age 6 years or older; diagnosis of Crohn's disease involving the colon, the small intestine, or both; and active luminal disease supported by a C-reactive protein (CRP) of more than 3 mg/L 90 days before the first dose, faecal calprotectin of more than 50 µg/g between 90 days before and 28 days after first dose, or both. Exclusion criteria included previous exposure to, or contraindications for the use of, anti-TNF therapy (all criteria available in the protocol).

The South West Research Ethics committee approved the study (REC reference: 12/SW/0323) in January, 2013. Patients were included after providing informed, written consent. The protocol is available online.

Procedures

The choice of anti-TNF was at the discretion of the treating physician and prescribed according to the licensed dosing schedule.

Study visits were scheduled at first dose (week 0), post-induction (week 14), and at weeks 30 and 54 after first dose. Additional visits were planned for infliximab-treated patients at each infusion, and for both groups at the time of treatment failure or treatment discontinuation. In cases in which the visit did not occur on the exact day delineated by the protocol, the following windows of eligibility were specified: week 0 (week –4 to 0), week 14 (week 10–20), week 30 (week 22–38), and week 54 (week 42–66; appendix pp 12–13).

At baseline, sites recorded demographic data, smoking status, age at diagnosis, disease duration, Montreal classification of disease location and behaviour,[12] previous medical and drug history, and previous Crohn's disease-related surgeries. At every visit, disease activity score, weight, therapy, and adverse events were recorded.

Blood and stool samples were processed through the central laboratory at the Royal Devon and Exeter National Health Service Foundation Trust. Drug and total anti-drug antibody concentrations were measured with IDKmonitor ELISA assays (Immundiagnostik AG, Bensheim, Germany) done on the Dynex DS2 ELISA robot (Dynex technologies, Worthing, UK; appendix p 11). For all infliximab-treated patients, we used trough drug concentrations, excluding concentrations measured at other timepoints. For adalimumab-treated patients, we asked research sites to take blood samples as near as possible to trough while minimising inconvenience to patients.

We chose a drug tolerant anti-drug antibody assay that allowed us to identify all patients with immunogenicity irrespective of circulating drug concentration. Based upon manufacturer's recommendation we defined immunogenicity as an anti-drug antibody titre of 10 arbitrary units per mL or more, and stratified immunogenicity by the presence or absence of detectable drug (<0·8 mg/L). Investigators were masked to these data until week 54.

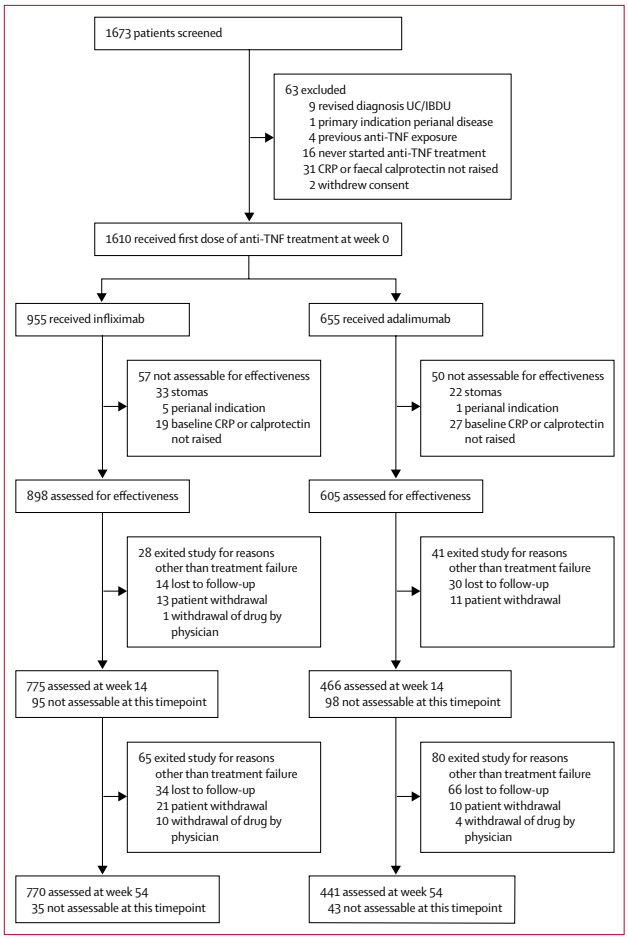

Figure 1: **Study profile**
Patients were not assessable when one or more key data items were missing. CRP=C-reactive protein. UC=ulcerative colitis. IBDU=inflammatory bowel disease unclassified.

Outcomes

Treatment failure endpoints were primary non-response at week 14, non-remission at week 54, and adverse events leading to drug withdrawal. We used composite endpoints defined using symptom scores (Harvey Bradshaw index [HBI] in adults[13] and the HBI or Short Paediatric Crohn's Disease Activity Index

For the **protocol** see https://www.ibdresearch.co.uk/pants/

For more on the **laboratory at the Royal Devon and Exeter NHS Foundation Trust** see https://www.exeterlaboratory.com/

	Infliximab	Adalimumab	p value
Sex	..	..	0·960
Male	465/955 (49%)	318/655 (49%)	..
Female	490/955 (51%)	337/655 (51%)	..
Age (years)	30 (20–45)	37 (28–50)	<0·0001
Ethnicity	..	..	0·00081
White	862/955 (90%)	624/655 (95%)	..
South Asian	49/955 (5%)	17/655 (3%)	..
Other	44/955 (5%)	14/655 (2%)	..
Disease duration (years)	2·3 (0·7–9·0)	3·3 (0·8–11·2)	0·0036
Age at diagnosis (years)	23·7 (15·7–33·8)	28·9 (21·4–40·6)	<0·0001
Montreal location classification*	..	..	0·022
L1	250/948 (26%)	214/644 (33%)	..
L2	239/948 (25%)	137/644 (21%)	..
L3	451/948 (48%)	287/644 (45%)	..
L4	8/948 (1%)	6/644 (1%)	..
Montreal L4 modifier	114/948 (12%)	32 (5%)	<0·0001
Montreal behaviour classification*	..	..	<0·0001
B1	591/948 (62%)	373/644 (58%)	..
B2	252/948 (27%)	229/644 (36%)	..
B3	105/948 (11%)	42/644 (7%)	..
Perianal disease	147/955 (15%)	52/655 (8%)	<0·0001
Immunomodulator	..	..	0·0035
Azathioprine	450/955 (47%)	265/655 (40%)	..
Mercaptopurine	78/955 (8%)	49/655 (7%)	..
Methotrexate	59/955 (6%)	30/655 (5%)	..
Tacrolimus	2/955 (<1%)	0	
None	366/955 (38%)	311/655 (47%)	..
Corticosteroids	274/955 (29%)	169/655 (26%)	0·21
Previous resectional surgery	207/955 (22%)	163/655 (25%)	0·15
HBI score	6 (3–9)	5 (3–8)	0·079
sPCDAI score	30 (15–50)	..	..
Body-mass index (kg/m^2)	22·7 (19·7–26·9)	24·4 (21·5–28·2)	<0·0001
Haemoglobin (g/L)	125 (114–135)	131 (120–142)	<0·0001
White cell count (×10^9 cells per L)	8·0 (6·1–10·3)	7·8 (6·1–9·9)	0·099
Platelet count (×10^9 cells per L)	343 (283–416)	311 (256–387)	<0·0001
Albumin (g/L)	39 (34–42)	39 (35–43)	0·0015
C-reactive protein (mg/L)	9 (3–24)	6 (2–14)	<0·0001
Faecal calprotectin (µg/g)	415 (164–862)	303 (134–634)	<0·0001

Data are number (%) or median (IQR). p values were calculated using Fisher's exact or Mann Whitney *U* tests. *Data are missing for seven patients treated with infliximab and 11 treated with adalimumab. HBI=Harvey-Bradshaw index. sPCDAI=short paediatric Crohn's disease index.

***Table 1*: Baseline demographic and clinical characteristics**

[sPCDAI] in children),[14] corticosteroid use, and CRP (appendix p 14).

Primary non-response was defined as exit before week 14 because of treatment failure (including resectional inflammatory bowel disease surgery) or corticosteroid use at week 14 (new prescriptions or if previous dose had not been stopped). Patients whose CRP did not decrease to 3 mg/L or less or by 50% or more from baseline (week 0), and whose HBI score did not decrease to 4 points or less or by 3 points or more from baseline, were also classified as having a primary non-response. Children were defined as having a primary non-response when their sPCDAI score did not decrease to 15 points or less or by more than 12·5 points from baseline (besides same CRP criteria as adults). Grey zone denoted an intermediate response between primary non-response and response, defined as CRP decreasing to 3 mg/L or less or by 50% or more from baseline (week 0), or HBI score decreasing to 4 points or less or by 3 points or more from baseline, but not both. Treatment response was defined as a decrease in CRP to 3 mg/L or less or by 50% or more from baseline (week 0) and a decrease in HBI to 4 points or less or by 3 points or more from baseline for adults, or a decrease in sPCDAI to 15 points or less or by 12·5 points from baseline (week 0) for children. Remission was defined as CRP of 3 mg/L or less and HBI score of 4 points or less (sPCDAI score ≤15 points), no ongoing steroid therapy, and no exit due to treatment failure. Loss of response in patients who did not have primary non-response was defined as symptomatic inflammatory bowel disease activity that warranted an escalation of steroid, immunomodulatory or anti-TNF therapy, resectional surgery, or exit from study due to treatment failure.[9] Timing of loss of response was defined as the time of treatment escalation, drug withdrawal, or surgery.

Non-remission was assessed at week 54 and defined as CRP of more than 3 mg/L or an HBI score of more 4 points (sPCDAI >15 points for children), ongoing steroid therapy, or exit due to treatment failure. Patients exited the study when they stopped anti-TNF therapy or had an intestinal resection. We defined steroid therapy for the purposes of non-remission and primary non-response as any systemic therapy, either oral or intravenous (including use of steroids for other conditions), but not including single pre-infusion dosing with hydrocortisone.

Adverse events were coded centrally according to the Medical Dictionary for Regulatory Activities (MedDRA) version 20.1. Serious adverse events included those that required hospitalisation, were life-threatening, or resulted in persistent, permanent, or substantial disability or incapacity. Causality was graded according to the Good Clinical Practice framework guidelines as not related, unlikely, possibly, probably, or definitely related to treatment by the local research sites.[15]

Statistical analysis

At cohort inception, sample size was based on the design of a genetic study aimed at identifying a genetic predictor of primary non-response, the results of which will be reported elsewhere. Assuming that 20% of patients would have a primary non-response, and assuming a perfectly tagged risk allele frequency of 25%, we calculated, using Purcell's genetic power calculator, that we needed to recruit 240 non-responders to yield 99% power to detect a genome-wide significant association ($p<5\times10^{-8}$) for a relative risk of 2, and 30% power for a relative risk of 1·5. We anticipated that the

	Infliximab		Adalimumab		Both drugs	
	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)
Week 14						
Primary non-response	170/775	21·9% (19·1–25·0)	125/466	26·8% (22·9–31·1)	295/1241	23·8% (21·4–26·2)
Grey zone	154/775	19·9% (17·1–22·9)	83/466	17·8% (14·4–21·6)	237/1241	19·1% (16·9–21·4)
Response	122/775	15·7% (13·2–18·5)	61/466	13·1% (10·2–16·5)	183/1241	14·7% (12·8–16·8)
Remission	329/775	42·5% (38·9–46·0)	197/466	42·3% (37·7–46·9)	526/1241	42·4% (39·6–45·2)
Week 54						
Non-remission	469/770	60·9% (57·4–64·0)	295/441	66·9% (62·3–71·3)	764/1211	63·1% (60·3–65·8)
Remission	301/770	39·1% (35·6–42·6)	146/441	33·1% (28·7–37·7)	447/1211	36·9% (34·2–39·7)
Adverse event curtailing treatment (not including exacerbation of Crohn's disease)	84/995	8·8% (7·1–10·8)	42/655	6·4% (4·7–8·6)	126/1610	7·8% (6·6–9·2)

Table 2: **Key outcomes at weeks 14 and week 54**

proportion of patients lost due to attrition would be 20%, so our recruitment target was 1600 patients.

In February, 2015, the infliximab biosimilar CT-P13 became available in the UK. We calculated that a sample size of 180 biosimilar-treated patients would permit a comparison of non-inferiority of biosimilar and originator infliximab based on a power of 80%, our observation that 25% of patients had a primary non-response, a non-inferiority margin of 10%, attrition rate of 20%, and a ratio of biosimilar-treated to originator infliximab-treated patients of 1:4.[12]

Following central monitoring, we identified three groups of patients who we subsequently excluded from the effectiveness analyses: patients with stomas because the HBI and sPCDAI are not validated for this patient group; patients that were recruited into the study with normal calprotectin and CRP concentrations at prescreening and during the first visit; and patients for whom the only indication for anti-TNF treatment was perianal disease. However, we included these patients in our immunogenicity and safety analyses, because they had received one of the drugs.

Because of differences in drug formulation, route of delivery, dosing interval, and potential for inducing immune response, infliximab and adalimumab treatment outcomes were analysed separately.[16] Outcomes were assigned using an algorithm written in R version 3.5.1. All analyses were two-tailed, and p values of less than 0·05 were considered significant.

Patients who exited the study because of treatment failure were deemed to be in non-remission for every subsequent timepoint. Patients who exited the study because of loss to follow-up, patient withdrawal of consent, or elective withdrawal of drug by their physician, including for pregnancy, were censored at the time of study exit and were excluded from the denominator for subsequent analyses.

We did univariable analyses using Fisher's exact and Mann-Whitney *U* tests to identify differences in baseline characteristics between infliximab-treated and adalimumab-treated patients, and to determine categorical and continuous factors associated with predefined outcomes. We used multivariable logistic regression analyses to identify which factors were independently associated with treatment failure. We included variables with a univariable p value of less than 0·05 in the model and used the Akaike information criterion (AIC) and backward stepwise variable selection. We also built predictive models, using forwards and backwards stepwise model selection starting from the null model (ie, with no covariates, just an intercept term), with AIC. We used leave-one-out cross-validation to test the model, firstly to ensure the model was not overfitted, and secondly to estimate the diagnostic accuracy of the model. For prediction testing, a probability threshold was determined by maximising the sum of sensitivity and specificity.

We explored associations with trough drug concentration using linear regression, using the same variable selection methods as those detailed for the logistic regression analyses. Proportions of patients with immunogenicity and loss or response were estimated using the Kaplan-Meier method, and comparative analyses were done by the use of univariable and multivariable Cox proportional hazards regression. For immunogenicity, patients were censored after their last drug and antibody measurement or at week 54. For loss of response, patients were censored if they exited for reasons other than treatment failure or at week 54.

Optimal thresholds for drug concentrations were determined graphically by plotting outcome against intervals of drug concentration and looking for the threshold beyond which further increases were not associated with improvement in outcome.

Non-inferiority for biosimilar infliximab was assessed by determining whether the one-sided 95% CI of the absolute difference in proportions was 10% or more. The confidence interval was calculated using the prop.test function in R software.

	Infliximab			Adalimumab		
	PNR	Not PNR	p value	PNR	Not PNR	p value
Age at first dose (years)	33·7 (22·5–49·1)	29·0 (18·4–42·5)	0·0048	38·6 (26·6–55·0)	36·3 (28·6–49·2)	0·30
Baseline immunomodulator	85/170 (50%)	392/605 (65%)	0·00067	58/125 (46%)	193/341(57%)	0·059
Baseline BMI category	..	..	0·29	..	..	0·0027
Normal	73/170 (43%)	291/605 (48%)	..	62/125 (50%)	164/341 (48%)	..
Underweight	31/170 (18%)	105/605 (17%)	..	5/125 (4%)	25/341 (7%)	..
Overweight	35/170 (21%)	132/605 (22%)	..	25/125 (20%)	106/341 (31%)	..
Obese	31/170 (18%)	77/605 (13%)	..	33/125 (26%)	46/341 (13%)	..
Baseline smoker	41/168 (24%)	84/598 (14%)	0·0020	25/124 (20%)	62/337 (18%)	0·69
Baseline white cell count ($\times 10^9$ cells per L)	8·9 (7·0–10·9)	7·9 (6·1–10·0)	0·0011	8·2 (6·5–10·4)	7·7 (6·1–9·6)	0·052
Baseline albumin (g/L)	37 (32–41)	39 (34–42)	0·0092	39 (34–43)	39 (36–43)	0·48
Week 14 drug concentration (mg/L)	2·3 (0·9–5·0)	4·0 (1·9–7·2)	0·00013	8·4 (4·4–11·3)	11·6 (8·4–15·3)	<0·0001
Week 14 anti-drug antibody concentration (AU/mL)	5·0 (3·0–9·0)	4·0 (2·0–6·0)	0·00039	3·0 (2·0–6·0)	2·0 (2·0–3·1)	0·0010

Data are median (IQR) or n (%). The significance of differences between continuous variables was calculated using the Mann-Whitney *U* test. Differences between categorical variables were sought using Fisher's exact test. Variables are only shown when the p value was less than 0·05 for either or both drugs. The full tables of variables tested are shown in the appendix. AU=arbitrary units. BMI=body-mass index. PNR=primary non-response.

***Table 3*: Significant univariable associations with primary non-response at week 14 in all participants**

This study was registered with ClinicalTrials.gov, number NCT03088449.

Role of the funding source

The funders of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between March 7, 2013, and July 15, 2016, 1610 patients were included in this prospective study; 955 (59%) patients were treated with infliximab (753 [47%] with originator infliximab, and 202 [13%] with biosimilar) and 655 (41%) were treated with adalimumab (figure 1). Differences between demographic and clinical characteristics of infliximab-treated and adalimumab-treated patients are shown in table 1 and the appendix (pp 34–35).

Several baseline characteristics were significantly different between the infliximab-treated and adalimumab-treated patients, including age, smoking, body-mass index, disease duration, disease location, and disease behaviour. Patients treated with infliximab had more active disease at baseline than did patients treated with adalimumab, as evidenced by higher serum CRP and faecal calprotectin concentrations (table 1). Most differences persisted when the 219 paediatric patients (aged <18 years at time of first dose) were excluded, almost all of whom were treated with infliximab (appendix p 34). At initiation of anti-TNF treatment, immunomodulator use was higher in patients treated with infliximab than those treated with adalimumab (589 [62%] of 955 *vs* 344 [53%] of 655; p<0·0001), but no differences were seen in the proportions of patients treated with corticosteroids (table 1).

1241 patients were assessable at week 14. Primary non-response occurred in 170 (21·9%, 95% CI 19·1–25·0) of 775 patients treated with infliximab and 125 (26·8%, 22·9–31·1) of 466 patients treated with adalimumab (table 2). After excluding primary non-responders, the estimated proportion of infliximab-treated patients who had loss of response by week 54 was 36·9% (32·7–40·9), and for adalimumab was 34·1% (28·4–39·4; appendix pp 15–16). At week 54, 469 (60·9%; 57·4–64·0) of 770 infliximab-treated patients were classified as being in non-remission, compared with 295 (66·9%; 62·3–71·3) of 441 adalimumab-treated patients (table 2).

Univariable analyses showed the strongest associations with primary non-response to infliximab and adalimumab were with week 14 drug and anti-drug antibody concentrations (table 3; appendix p 17). Primary non-response to infliximab was also associated with older age at first dose, smoking at baseline, non-use of an immunomodulator at baseline, lower baseline albumin concentrations, and higher baseline white cell count. Primary non-response to adalimumab was associated with a higher body-mass index at baseline.

Univariable analysis showed, for both drugs, that the most significant determinant of non-remission at week 54 was clinical status at week 14 (table 4; appendix pp 21–22). Despite meeting primary non-response criteria, 76 (44·7%, 95% CI 37·1–52·5) of 170 infliximab-treated patients and 61 (48·8%, 39·8–57·9) of 125 adalimumab-treated patients continued drug beyond week 20. Of these, only ten (14·9%, 7·4–25·7) of 67 patients treated with infliximab (data for nine patients continuing infliximab after primary non-response not available) and four (8·7%, 2·4–20·8) of

	Infliximab			Adalimumab		
	Non-remission	Remission	p value	Non-remission	Remission	p value
Sex			0·0025			0·32
Female	198/364 (54%)	118/279 (42%)	..	99/214 (46%)	68/130 (52%)	..
Male	166/364 (46%)	161/279 (58%)	..	115/214 (54%)	62/130 (48%)	..
Age at first dose (years)	32·0 (21·1–46·2)	27·0 (17·6–38·9)	0·00043	38·9 (29·2–51·5)	36·1 (27·6–51·0)	0·32
Baseline immunomodulator	196/364 (54%)	209/279 (75%)	<0·0001	112/214 (52%)	75/130 (58%)	0·37
History of perianal disease	50/364 (14%)	56/279 (20%)	0·041	9/214 (4%)	14/130 (11%)	0·025
Baseline BMI category	..	..	0·00022	..	..	<0·0001
Normal	153/364 (42%)	144/279 (52%)	..	89/214 (42%)	81/130 (62%)	..
Underweight	58/364 (16%)	60/279 (22%)	..	10/214 (5%)	10/130 (8%)	..
Overweight	89/364 (24%)	53/279 (19%)	..	67/214 (31%)	29/130 (22%)	..
Obese	64/364 (18%)	22/279 (8%)	..	48/214 (22%)	10/130 (8%)	..
Baseline current smoker	64/359 (18%)	30/278 (11%)	0·013	47/212 (22%)	15/129 (12%)	0·014
Baseline white cell count (×10^9 cells per L)	8·6 (6·6–10·8)	7·4 (5·7–9·5)	<0·0001	8·3 (6·3–9·8)	7·5 (5·7–10·2)	0·15
Week 14 drug concentration (mg/L)	2·9 (1·2–5·7)	5·3 (2·8–8·8)	<0·0001	9·2 (7·0–12·5)	13·3 (10·7–17·8)	<0·0001
Week 14 anti-drug antibody concentration (AU/mL)	4·0 (3·0–9·0)	3·0 (2·0–5·0)	<0·0001	3·0 (2·0–4·0)	2·0 (2·0–3·0)	0·011
Week 14 status	..	..	<0·0001	..	..	<0·0001
Remission	104/353 (29%)	188/266 (71%)	..	63/202 (31%)	94/127 (74%)	..
Response	78/353 (22%)	28/266 (11%)	..	37/202 (18%)	12/127 (9%)	..
Grey zone	100/353 (28%)	40/266 (15%)	..	50/202 (25%)	17/127 (13%)	..
PNR	71/353 (20%)	10/266 (4%)	..	52/202 (26%)	4/127 (3%)	..
Immunogenicity in first year	..	..	<0·0001	..	..	0·012
Antibody negative	137/364 (38%)	142/279 (51%)	..	155/210 (74%)	112/130 (86%)	..
Antibody positive, drug positive	94/364 (26%)	97/279 (35%)	..	37/210 (18%)	15/130 (12%)	..
Antibody positive, drug negative	133/364 (37%)	40/279 (14%)	..	18/210 (9%)	3/130 (2%)	..

Data are median (IQR) or n (%). The significance of differences between continuous variables was calculated using the Mann-Whitney *U* test. Differences between categorical variables were sought using Fisher's exact test. Variables are only shown when the p value was less than 0·05 for either or both drugs. The full tables of variables tested are shown in the appendix. AU=arbitrary units. BMI=body-mass index. PNR=primary non-response.

Table 4: **Significant univariable associations with non-remission at week 54, excluding participants who exited due to primary non-response**

46 patients treated with adalimumab (data for 15 patients continuing adalimumab after primary non-response not available) were in remission at week 54 (14 [12·4%, 95% CI 6·9–19·9] of 113 patients overall). Body-mass index, baseline smoking status, week 14 drug concentration, week 14 antibody concentration, and immunogenicity in first year were also associated with non-remission at week 54 for both drugs. In addition, among patients treated with infliximab, but not those treated with adalimumab, non-remission at week 54 was associated with older age, female sex, non-use of an immunomodulator at baseline, and higher baseline white cell count.

Multivariable analyses showed that, for both drugs, only week 14 drug concentration was independently associated with primary non-response (table 5). A dose–response association was seen for week 14 drug concentration and remission up to 7 mg/L for infliximab and 12 mg/L for adalimumab (appendix p 18). In infliximab-treated patients for whom we measured drug concentrations at week 6, a dose–response association was seen between week 6 drug concentrations up to 30–35 mg/L and increasing week 14 remission (appendix p 19). Our predictive models of primary non-response to infliximab and adalimumab, however, were not clinically useful (appendix pp 20, 37). For infliximab, the area under the curve (AUC) was 0·53 (95% CI 0·46–0·59) with a sensitivity of 0·35, specificity of 0·75, positive predictive value of 0·27, and negative predictive value of 0·81, whereas for adalimumab the AUC was 0·54 (0·46–0·62) with a sensitivity of 0·35, specificity of 0·95, positive predictive value of 0·56, and negative predictive value of 0·78.

Among patients who continued treatment beyond week 14, multivariable analyses (table 5) showed, independent associations between drug concentrations at week 14 and non-remission at week 54 for both drugs. The optimal drug concentration at week 14 that was associated with remission at week 54 was 7 mg/L for infliximab and 12 mg/L for adalimumab (appendix p 18). Obesity at baseline was associated with non-remission at week 54 only in patients treated with adalimumab. Smoking at baseline and no previous history of perianal

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	Infliximab		Adalimumab	
	OR*, fold-change†, or HR‡ (95% CI)	p value	OR*, fold-change†, or HR‡ (95% CI)	p value
Primary non-response at week 14*				
Baseline immunomodulator	0·71 (0·44–1·13)	0·14	..	..
Log_{10} (week 14 drug concentration [mg/L])	0·35 (0·20–0·62)	0·00038	0·13 (0·06–0·28)	<0·0001
Baseline BMI category				
Normal	..	..	1 (ref)	..
Underweight	..	..	0·36 (0·07–1·20)	0·13
Overweight	..	..	0·63 (0·34–1·15)	0·14
Obese	..	..	1·57 (0·82–2·99)	0·17
Week 54 non-remission*				
Sex				
Male	0·68 (0·45–1·02)	0·063	..	..
Female	1 (ref)	1 (ref)	..	..
Age at first dose (years)	1·01 (1·00–1·03)	0·11	..	..
History of perianal disease	0·60 (0·35–1·04)	0·070	0·29 (0·09–0·85)	0·029
Baseline BMI category				
Normal	1 (ref)	..	1 (ref)	..
Underweight	0·92 (0·51–1·65)	0·79	1·20 (0·40–3·57)	0·74
Overweight	1·73 (1·02–2·99)	0·045	2·31 (1·28–4·25)	0·0059
Obese	1·99 (0·98–4·17)	0·062	3·42 (1·51–8·43)	0·0046
Baseline white cell count ($\times 10^9$ cells per L)	1·05 (0·99–1·12)	0·12	..	..
Log_{10} (week 14 drug concentration [mg/L])	0·29 (0·16–0·52)	<0·0001	0·03 (0·01–0·12)	<0·0001
Immunogenicity in first year				
Antibody negative	1 (ref)	..	..	..
Antibody negative, detectable drug concentration	0·77 (0·48–1·22)	0·27	..	..
Antibody positive, undetectable drug concentration	1·64 (0·95–2·85)	0·079	..	..
Smoker at baseline	..	..	2·27 (1·13–4·81)	0·025
Week 14 drug concentration†				
Log_{10} (baseline faecal calprotectin [µg/g])	0·81 (0·68–0·98)	0·028	..	..
Smoker at baseline	0·78 (0·61–0·99)	0·045	0·89 (0·77–1·03)	0·12
Log_{10} (week 14 anti-drug antibody concentration [mg/L])	0·50 (0·42–0·60)	<0·0001	0·40 (0·35–0·45)	<0·0001
Log_{10} (week 14 CRP [mg/L])	0·74 (0·62–0·88)	0·00075	..	..
Week 14 albumin (g/L)	1·03 (1·01–1·05)	0·00018	..	..
Log_{10} (week 14 faecal calprotectin [µg/g])	0·74 (0·63–0·88)	0·00057	0·72 (0·66–0·79)	<0·0001
Baseline BMI category				
Normal	..	..	1 (ref)	..
Underweight	..	..	1·07 (0·84–1·36)	0·57
Overweight	..	..	0·88 (0·78–1·00)	0·056
Obese	..	..	0·71 (0·60–0·83)	<0·0001
Baseline HBI or sPCDAI remission	..	..	1·14 (1·02–1·28)	0·021
Log_{10} (baseline CRP [mg/L])	..	..	0·91 (0·82–1·00)	0·056

(Table 5 continues on next page)

disease were associated with poorer outcomes at week 54 for both drugs on univariable analyses, but only for adalimumab on multivariable analyses (data not shown for infliximab because smoking at baseline dropped out of the model during backwards stepwise regression).

We devised two diagnostic models informed by significant univariable factors to predict non-remission to infliximab and adalimumab at week 54. Our first model attempted to predict non-remission using baseline variables only and had low diagnostic value. Our second model using baseline variables and week 14 pharmacokinetic data had greater predictive power: for infliximab, the AUC was 0·814 (95% CI 0·76–0·87) and for adalimumab 0·75 (0·68–0·83; appendix pp 20, 39–41).

Serious adverse events, excluding worsening of Crohn's disease activity, were observed in 171 (17·9%, 95% CI 15·5–20·5) of 955 infliximab-treated patients and 96 (14·7%, 12·0–17·6) of 655 adalimumab-treated patients. Adverse events leading to treatment withdrawal occurred in 84 (8·8%, 7·1–10·8) of 955 patients treated with infliximab and 42 (6·4%, 4·7–8·6) of 655 patients treated with adalimumab (appendix p 42).

Five patients died (three treated with infliximab and two with adalimumab), all of whom were in the upper quartile for age. None of those who died had responded to treatment by the time of death: four died from sepsis (two from pneumonia, two from intra-abdominal sepsis), and one of Crohn's disease-related malnutrition. Four of the five patients were taking concomitant corticosteroids at the time of death and one was taking azathioprine.

Serious infections were reported in 38 (4·0%, 95% CI 2·8–5·4) of 955 infliximab-treated patients, including active tuberculosis in three patients, and 21 (3·2%, 2·0–4·9) of 655 adalimumab-treated patients, none of whom had tuberculosis (appendix p 42). Concomitant immunomodulatory therapy was not associated with an increased risk of infections, even when stratified by age (appendix p 43).

Infusion reactions within 24 h of infliximab, which occurred after a median of 5 weeks (IQR 1–14) of starting treatment, were observed in 31 (3·2%, 95% CI 2·3–4·6) of 955 patients (appendix p 42) and were associated with anti-drug antibody titre (median peak antibody 96 arbitrary units per mL [IQR 5–313] in patients with an infusion reaction *vs* 8 arbitrary units per mL [5–45] in patients without an infusion reaction; p=0·0037). Injection site reactions to adalimumab, which occurred after a median of 14 weeks (IQR 3·5–27·2), were observed in 28 (4·3% [95% CI 2·9–6·2]) of 655 patients (appendix p 42) but were not associated with immunogenicity (p=0·58).

Univariable factors associated with low drug concentrations at weeks 14 and 54 are shown in the appendix (pp 23, 24, 44, 45). In multivariable analyses, for both drugs, low drug concentrations at both week 14 and week 54 were significantly associated with week 14

anti-drug antibody formation and markers of active disease (table 5). Lower albumin concentrations at week 14 were independently associated with week 14 drug concentration for infliximab, whereas obesity at baseline was independently associated with week 14 and week 54 drug concentrations for adalimumab. In patients treated with infliximab but not those treated with adalimumab, use of an immunomodulator at baseline was associated with higher week 54 drug concentrations (table 5).

The estimated proportion of patients with immunogenicity by week 54 was 62·8% (95% CI 59·0–66·3) for infliximab-treated patients and 28·5% (24·0–32·7) for adalimumab-treated patients (appendix p 25). 31·2% (95% CI 27·6–34·6) of patients treated with infliximab, and 12·3% (8·9–15·6) of those treated with adalimumab had anti-drug antibody concentrations of 10 arbitrary units per mL or more and undetectable drug concentrations at week 54 (appendix p 27).

Among infliximab-treated patients for whom early anti-drug antibody concentrations were available, the Kaplan-Meier estimate of anti-drug antibody positivity was 1·6% (95% CI 0·8–2·4) at 2 weeks, 3·3% (2·2–4·5) at 6 weeks, and 17·2% (14·6–19·7) at 14 weeks.

The univariable factors associated with time to immunogenicity are shown in figure 2 and the appendix (p 46), respectively. Multivariable analyses showed that drug concentration at week 14 was the major independent risk factor associated with time to immunogenicity for both drugs after that timepoint. In addition, time to immunogenicity was associated with obesity for adalimumab-treated patients and smoking for infliximab-treated patients (figure 2, table 5).

Immunomodulator use was the main protective factor against immunogenicity, with similar effect sizes for infliximab (hazard ratio [HR] 0·39 [95% CI 0·32–0·46], p<0.0001) and adalimumab (HR 0·44 [0·31–0·64], p<0.0001) (appendix p 46). No difference was measured in time to immunogenicity between thiopurine medications or methotrexate (appendix pp 29–30). Thiopurines reduced immunogenicity to infliximab in a dose-dependent manner with the lowest immunogenicity observed in patients treated with the highest thiopurine doses (appendix p 31).

Sensitivity analyses exploring the effect of combination immunomodulator use on clinical outcomes showed that immunomodulator use was associated with a lower proportion of infliximab-treated patients in non-remission at week 54 than was monotherapy (combination 52·6% [95% CI 47·9–57·1] *vs* monotherapy 74·0% [68·6–78·9]), but this association was not seen in adalimumab-treated patients (64·2% [57·6–70·4] *vs* 69·8% [63·1–75·9]). Further sensitivity analyses of infliximab-treated patients, limited to the modifiable factors of immunomodulator use and drug and anti-drug antibody concentrations, showed that the benefit of immunomodulators (odds ratio [OR] 0·56 [95% CI 0·38–0·83]) was independent of $\log_{10}$ drug concentration (0·30 [0·18–0·49]) or $\log_{10}$ anti-drug antibody concentration (1·61 [1·02–2·63]) status at week 14 (appendix p 47).

No differences were measured in baseline demographic or clinical characteristics between patients treated with biosimilar and originator infliximab (appendix p 48). Of 955 patients treated with originator infliximab, 79 (8%) changed to biosimilar during the first year of treatment and were excluded from analyses comparing originator and biosimilar infliximab after the switch date. At week 14, biosimilar was non-inferior to originator infliximab for primary non-response (difference in proportions –3·9% [one-sided 95% CI upper bound 2·4]). At week 54, biosimilar was non-inferior to originator infliximab for non-remission (–2·2% [one-sided 95% CI upper bound 5·6]; appendix p 32). Among patients who started on originator infliximab and did not switch during the first

	Infliximab		Adalimumab	
	OR*, fold-change†, or HR‡ (95% CI)	p value	OR*, fold-change†, or HR‡ (95% CI)	p value
(Continued from previous page)				
Week 54 drug concentration†				
Baseline immunomodulator	1·27 (1·02–1·59)	0·034	..	..
Baseline BMI category				
Normal	1 (ref)	..	1 (ref)	..
Underweight	0·80 (0·62–1·04)	0·092	1·02 (0·80–1·30)	0·85
Overweight	0·79 (0·62–1·00)	0·048	0·88 (0·77–0·99)	0·041
Obese	0·95 (0·67–1·35)	0·77	0·73 (0·63–0·85)	<0·0001
$\log_{10}$ (week 14 anti-drug antibody concentration [mg/L])	0·73 (0·58–0·92)	0·0087	0·40 (0·35–0·44)	<0·0001
$\log_{10}$ (week 14 CRP [mg/L])	0·88 (0·72–1·07)	0·19	..	..
$\log_{10}$ (week 14 faecal calprotectin [µg/g])	0·74 (0·62–0·89)	0·00099	0·72 (0·66–0·79)	<0·0001
Week 14 HBI or sPCDAI remission	..	..	1·25 (1·10–1·41)	0·00066
Smoker at baseline	..	..	0·90 (0·79–1·04)	0·16
Immunogenicity‡				
Disease duration	0·99 (0·98–1·01)	0·32	..	..
Baseline immunomodulator	0·39 (0·32–0·48)	<0·0001	0·47 (0·32–0·67)	<0·0001
Baseline BMI category				
Normal	1 (ref)	..	1 (ref)	..
Underweight	1·29 (0·99–1·69)	0·060	1·26 (0·60–2·67)	0·54
Overweight	0·94 (0·74–1·20)	0·61	1·08 (0·70–1·67)	0·73
Obese	1·27 (0·96–1·67)	0·090	2·23 (1·44–3·46)	<0·0001
Current smoker	1·42 (1·12–1·81)	0·0045	..	..
Immunogenicity (excluding patients with immunogenicity or censored before week 14)				
Baseline immunomodulator	0·57 (0·43–0·75)	<0·0001	0·39 (0·22–0·69)	0·0011
Smoker at baseline	1·88 (1·35–2·62)	0·00021	..	..
$\log_{10}$ (week 14 drug concentration [mg/L])	0·43 (0·30–0·61)	<0·0001	0·05 (0·02–0·14)	<0·0001

BMI=body-mass index. CRP=C-reactive protein. HBI=Harvey-Bradshaw index. sPCDAI=Short Paediatric Crohn's Disease Activity Index. *ORs (95% CI) and p values were calculated using logistic regression for week 14 primary non-response and week 54 non-remission. †For drug concentration, models were calculated using linear regression of the log-transformed drug concentration, with exponentiated β values expressed here as fold changes (95% CI). ‡For immunogenicity, models were generated using Cox proportional hazards and coefficients expressed as hazard ratios (HRs [95% CI]).

Table 5: **Multivariable analyses of treatment failure outcomes**

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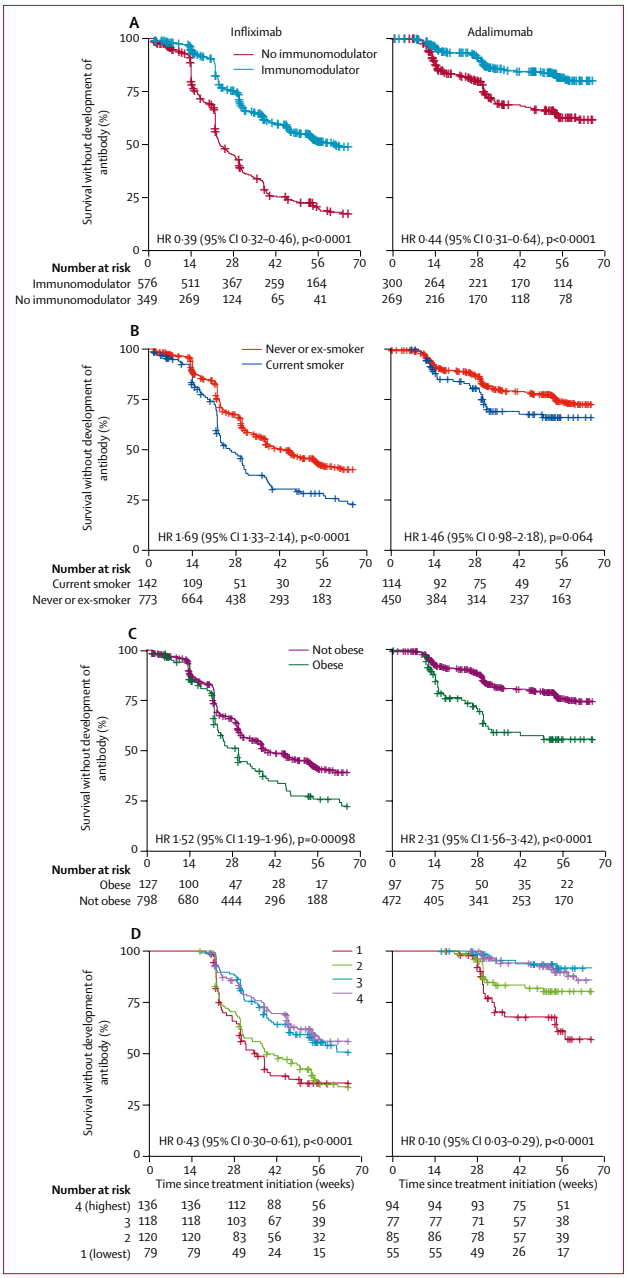

year, 64 (9%) of 674 exited the study because of adverse events; among patients treated with biosimilar infliximab, 16 (8%) of 202 exited for adverse events (one-sided 95% CI upper bound of difference 5·5%). The estimated proportion of patients with immunogenicity by week 54 was 62·1% (95% CI 57·4–66·2) for patients treated with originator infliximab and 64·5% (55·4–71·7) for patients treated with biosimilar infliximab (appendix p 25). 31·3% (27·0–35·4) of patients treated with originator infliximab and 33·5% (25·0–41·0) of those treated with biosimilar had anti-drug antibody concentrations of 10 arbitrary units per mL or more and undetectable drug concentrations at week 54 (appendix p 27).

Discussion

Our cohort study of 1610 anti-TNF-naive patients with active luminal Crohn's disease showed that primary non-response occurred in 24% and non-remission in 63% of patients, and that adverse events curtailed treatment in 8% of patients. Obesity, smoking, low albumin concentrations, higher baseline markers of disease activity, and development of immunogenicity were all associated with low drug concentrations, which mediated non-remission.

Numerous studies have reported an association between drug concentration and clinical outcome, although the therapeutic thresholds, particularly for adalimumab, are poorly defined.[17] In our study, low drug concentrations during induction were associated with primary non-response at week 14 and non-remission at week 54. Patients with primary non-response who continued standard dosing regimens rarely entered remission. Dose intensification might improve outcomes for patients with suboptimal drug concentrations at week 14, whereas an early switch out-of-class might be more appropriate for patients with optimal drug concentrations. Despite variation in drug concentration among patients in remission, our data suggest that a higher target drug concentration might be required during induction than those reported in previous studies,[18] probably reflecting our more stringent definition of remission. The optimal week 14 drug concentrations associated with remission at both week 14 and 54 were 7 mg/L for infliximab and 12 mg/L for adalimumab. The importance of drug concentration is further shown by our predictive models, which were

Figure 2: **Univariable associations of time to immunogenicity using Kaplan-Meier and Cox proportional hazards methods**
Kaplan-Meier graphs for survival without development of any antibody (defined as 10 arbitrary units per mL or more) according to baseline immunomodulator use (A), smoking status (B), body-mass index category (C), and week 14 drug concentration quartile (with 1 being the lowest and 4 being the highest) (D). p values and HRs are derived from Cox proportional hazards models for each individual variable. The data for week 14 drug quartile excludes anyone who developed immunogenicity or exited the study before week 14, and is based on the $\log_{10}$ of the drug concentration. Therefore, the data show the HR for each ten-fold increase. HR=hazard ratio.

only clinically useful when week 14 pharmacokinetic data were included.

Previous prospective randomised studies of proactive dose increases based on drug concentrations did not show improved clinical outcomes. For both the TAXIT[19] and TAILORIX[20] studies, this absence of improvement might in part be explained by inclusion of patients after the crucial induction period and use of infliximab thresholds of 3 mg/L. Further adequately powered clinical trials are required to investigate whether optimising drug concentration on a treat-to-target basis during the induction period improves outcomes.[21]

Using analytical platforms that differed only by target antibody, we have shown that immunogenicity is more common in patients treated with infliximab than adalimumab: an observation frequently attributed to the chimeric formulation of infliximab. For both drugs, however, we observed a bidirectional negative relationship between drug concentration and immunogenicity. The lowest drug concentrations were measured in patients with high titre anti-drug antibody concentrations, in keeping with the known effect of the antibodies on drug clearance. Conversely, low drug concentrations at week 14 were associated with an increased risk of immunogenicity by week 54. This association is consistent with the discontinuity theory of the immune response, which proposes that intermittent exposure to antigen promotes a persistent immune reaction, whereas exposure at constant concentrations, observed with adalimumab delivered subcutaneously every 2 weeks, induces an immune tolerance.[22] Immunogenicity, which we have shown might occur earlier than previously suggested by other studies, might be mitigated by early dose optimisation, minimising loss of response.[23] We accept, however, that this observation might be explained by the formation of anti-drug antibodies at concentrations sufficient to lower the drug concentration but not detectable by our assay.

Immunomodulator use was associated with lower immunogenicity to both drugs and higher drug concentrations for infliximab-treated patients compared with no immunomodulator use. Methotrexate exerted a similar effect to thiopurine drugs on immunogenicity. In contrast to previous reports,[24,25] we showed that thiopurines reduced immunogenicity in infliximab-treated patients in a dose-dependent manner without an obvious threshold effect. Post-hoc analyses of the SONIC study suggested that the primary benefit of azathioprine was on pharmacokinetics of infliximab.[26] Conversely, in our study, we showed that concomitant immunomodulator use in infliximab-treated patients was associated with higher week 54 remission compared with no immunomodulator use, independently of week 54 drug concentration or immunogenicity status, suggesting that the addition of immunosuppression to anti-TNF therapy might have additional benefits. Consistent with previous studies,[27] immunomodulator use was not associated with increased remission for adalimumab treatment; however, this finding might have been influenced by low rates of immunogenicity, short duration of follow-up, or both.

We have shown that obesity is independently associated with low drug concentrations and non-remission at week 54 for adalimumab. Our data suggest that the previously reported associations of obesity and primary non-response are likely to be mediated by low drug concentrations.[28] For adalimumab-treated patients, fixed dosing was probably a major contributing factor. Obesity was also associated with immunogenicity to adalimumab; further clinical trials of dose optimisation are needed to clarify if this finding was because of suboptimal dosing during induction or whether obesity contributes to immunogenicity directly.

Our observation that cigarette smoking was independently associated with an increased risk of immunogenicity to infliximab might explain the poorer, less durable anti-TNF response reported in patients with Crohn's disease who smoke than in non-smokers.[29]

Previous studies investigating the association between baseline markers of inflammation and anti-TNF response are conflicting.[2,30] In our study, higher baseline markers of inflammation predicted lower drug concentrations at week 14, suggesting that higher inflammatory load might contribute to faster drug elimination. We have shown that lower baseline albumin concentrations predict suboptimal week 14 infliximab concentrations, similarly to other studies.[31] This association might reflect increased drug clearance as well as higher faecal protein losses.

Data from a nationwide population-based study suggest that benefits of anti-TNF and immunomodulatory combination treatment need to be considered against the additional risks of serious and opportunistic infection.[32] In this study, while acknowledging our smaller sample size and shorter duration of follow-up, combination therapy was not associated with an increased risk of infection in the first year of treatment, even among older patients (>50 years). However, sepsis was the cause of death in four of the five patients who died in the first year: all were older than 50 years, all but one was prescribed concomitant corticosteroids, and none had responded to anti-TNF.

Our study had some limitations. We used pragmatic definitions of treatment ineffectiveness combining corticosteroid use with clinical and biochemical markers of disease activity that are closely aligned with routine treatment targets. Although we accept that our data would have been strengthened by endoscopic outcomes, we observed a significant association between clinical outcomes at weeks 14 and week 54 and faecal calprotectin (appendix p 33). We are likely to have underestimated the rate of loss of response because our definition required an increase in therapy that was not always initiated. In addition, we used a pragmatic schedule of visits to minimise inconvenience to patients, and fewer assessments were undertaken for adalimumab-treated than infliximab-treated patients. We acknowledge that because CRP is

elevated in obesity we might have overestimated the effect of body-mass index on treatment ineffectiveness. Finally, we did not do real-time monitoring; therefore, the proportion of missing data is higher in this study than in registration trials.[6–8]

To our knowledge, this study is the largest prospective study of anti-TNF therapy in inflammatory bowel disease. We have shown that the major modifiable factors associated with treatment ineffectiveness were low drug concentrations and immunogenicity. Concomitant immunomodulator use and dose intensification in at-risk individuals during induction might improve the effectiveness and durability of treatment. Reassuringly, treatment ineffectiveness, safety, and immunogenicity of originator infliximab are no different to the biosimilar, which removes some of the cost constraints of dose intensification. Further clinical trials are required to better understand whether these strategies can allow us to improve the effectiveness and durability of anti-TNF therapy.

Contributors

TA, CB, GAH, JRFC, ALH, MP, JCM, PMI, JL, RKR, and CWL participated in the conception and design of this study. CB was the project manager and coordinated patient recruitment. TJM, MHP, and RN coordinated all biochemical analysis and central laboratory aspects of the project. NAK, JRG, TA, GAH, JRFC, CPS, CWL, ALH, MP, SS, JCM, PMI, JL, RKR, PH, NMH, DM, AT, GJW, NC, SL, SB, and DRG were involved in the acquisition, analysis, or interpretation of data. Data analysis was done by NAK, GAH, HDG, and BH. Drafting of the manuscript was done by TA, JRG, NAK, DM, GAH, TM, CB, NC, and SL. TA obtained the funding for the study. All the authors contributed to the critical review and final approval of the manuscript.

Declaration of interests

NAK declares personal fees from Falk, Takeda, and Pharmacosmos; other fees from Janssen; and non-financial support from Janssen, AbbVie, and Celltrion outside the submitted work. GAH reports non-financial support from AbbVie, outside the submitted work, and is now an employee of AbbVie and owns stock in the company. GJW reports grants from Crohn's Colitis UK; and personal fees from AbbVie, Janssen, Tillotts, and Falk, outside the submitted work. AH has served as consultant, advisory board member, or speaker for AbbVie, Atlantic, Bristol-Myers Squibb, Celltrion, Falk, Ferring, Janssen, MSD, Napp Pharmaceuticals, Pfizer, Pharmacosmos, Shire, and Takeda; and also serves on the Global Steering Committee for Genentech, all outside the submitted work. SS reports personal fees from AbbVie, Merck, and Takeda; and grants from Takeda, Tillotts, AbbVie, and Merck, outside the submitted work. PMI reports personal fees from AbbVie, Janssen, Pfizer, Sandoz, VH squared, Samsung Bioepis, and Ferring; grants from MSD; grants and personal fees from Takeda; and non-financial support from Falk, outside the submitted work. DM reports grants from the US National Institutes of Health and Helmsley Charitable Trust, during this study; and grants and personal fees from Janssen, Precision IBD Inc, Second Genome, Qu Biologics, Pfizer, Gilead, and Takeda, outside the submitted work. CWL reports grants and personal fees from AbbVie, GlaxoSmithKline, Janssen, Takeda, Amgen, Pfizer, Samsung Bioepis, and Cellgene; and grants and personal fees from Gilead, outside the submitted work. JRFC reports personal fees from AbbVie, Janssen, MSD, Celltrion, Napp Pharmaceuticals, and Sandoz; and grants and personal fees from Takeda, Biogen, and Hospira, outside the submitted work. CPS received grants from Warner Chilcott and Abbvie; has provided consultancy to Warner Chilcott, Dr Falk, AbbVie, Takeda, and Janssen; and had speaker arrangements with Warner Chilcott, Dr Falk, AbbVie, MSD, and Takeda. JL has served as advisory board member for Atlantic Health, AbbVie, MSD, Shire, Ferring International, Celltrion, Takeda, Pfizer, Janssen, GlaxoSmithKline; has served as a consultant for AbbVie UK, Takeda, Bristol-Myers Squibb; has received grants from Takeda, Hospira (Pfizer), AbbVie, Global, Ferring, MSD, Allergen, Shire, Cornerstone US, and Janssen; fees for educational presentations from AbbVie International and Cornerstone UK; travel and accommodation expenses from AbbVie, Warner Chilcot UK, and Takeda; and has received travel support from AbbVie, Warner Chilcot, Takeda, and Pfizer, outside the submitted work. RKR reports honoraria from Abbvie, Ferring, Therakos, and Celltrion; and grants from Nestec, outside the submitted work. JRG received honoraria from Falk, AbbVie, and Shield therapeutics, outside the submitted work for unrelated topics. TA reports grants from AbbVie, MSD, Napp Pharmaceuticals, Celltrion, Pfizer, Janssen, and Celgene during this study; personal fees and non-financial support from Immunodiagnostik; personal fees and non-financial support from Napp Pharmaceuticals, AbbVie, and MSD; personal fees from Celltrion and Pfizer; grants and personal fees from Takeda; and grants and non-financial support from Tillotts, outside the submitted work. All other authors declare no competing interests.

Acknowledgments

Laboratory tests were undertaken by the Exeter Blood Sciences Laboratory at the Royal Devon and Exeter National Health Service (NHS) Trust. The Exeter National Institute for Health Research (NIHR) Clinical Research Facility coordinated sample storage and management. The sponsor of the study was the Royal Devon and Exeter NHS Foundation Trust. TJM is funded by an NIHR Health Education England (HEE) Senior Lectureship. The views expressed in this publication are our own and not necessarily those of the NHS, HEE, NIHR, or the UK Department of Health. We are grateful to the following for their participation in our interim data interpretation meeting: Shomron Ben-Horin, Yehuda Chowers, Geert D'Haens, John Ding, and James Lee. We thank the patients who participated in this study, the Inflammatory Bowel Disease Pharmacogenetic Study Group, and research nurses who collected clinical data and biological samples at each study visit. We would also like to acknowledge the study co-ordinators of the Exeter Inflammatory Bowel Disease Research Group: Marian Parkinson and Helen Gardner-Thorpe for their ongoing administrative support to the study.

References

1 Lichtenstein GR, Yan S, Bala M, Blank M, Sands BE. Infliximab maintenance treatment reduces hospitalizations, surgeries, and procedures in fistulizing Crohn's disease. *Gastroenterology* 2005; **128:** 862–69.

2 Colombel JF, Sandborn WJ, Reinisch W, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362:** 1383–95.

3 D'haens G, Van Deventer S, Van Hogezand R, et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: a European multicenter trial. *Gastroenterology* 1999; **116:** 1029–34.

4 Louis E, Löfberg R, Reinisch W, et al. Adalimumab improves patient-reported outcomes and reduces indirect costs in patients with moderate to severe Crohn's disease: results from the CARE trial. *J Crohns Colitis* 2013; **7:** 34–43.

5 Loftus E V, Feagan BG, Colombel J-F, et al. Effects of adalimumab maintenance therapy on health-related quality of life of patients with Crohn's disease: patient-reported outcomes of the CHARM trial. *Am J Gastroenterol* 2008; **103:** 3132–41.

6 Rutgeerts P, D'Haens G, Targan S, et al. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 1999; **117:** 761–69.

7 Hanauer SB, Sandborn WJ, Rutgeerts P, et al. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. *Gastroenterology* 2006; **130:** 323–33.

8 Colombel J-F, Sandborn WJ, Rutgeerts P, et al. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132:** 52–65.

9 Ben-Horin S, Chowers Y. Review article: loss of response to anti-TNF treatments in Crohn's disease. *Aliment Pharmacol Ther* 2011; **33:** 987–95.

10 Sprakes MB, Ford AC, Warren L, Greer D, Hamlin J. Efficacy, tolerability, and predictors of response to infliximab therapy for Crohn's disease: a large single centre experience. *J Crohns Colitis* 2012; **6:** 143–53.

11 Naviglio S, Giuffrida P, Stocco G, et al. How to predict response to anti-tumour necrosis factor agents in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol* 2018; **12:** 797–810.

12 Chow S-C, Shao J, Wang H. Sample size calculations in clinical research. New York, NY: Marcel Dekker, 2003.

13 Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1980; **1:** 514.

14 Kappelman MD, Crandall W V, Colletti RB, et al. Short pediatric Crohn's disease activity index for quality improvement and observational research. *Inflamm Bowel Dis* 2011; **17:** 112–17.

15 WHO. The use of the WHO-UMC system for standardised case causality assessment-1-The use of the WHO-UMC system for standardised case causality assessment. https://www.who.int/medicines/areas/quality_safety/safety_efficacy/WHOcausality_assessment.pdf (accessed Dec 18, 2018).

16 Kugathasan S, Denson LA, Walters TD, et al. Prediction of complicated disease course for children newly diagnosed with Crohn's disease: a multicentre inception cohort study. *Lancet* 2017; **389:** 1710–18.

17 Vande Casteele N, Herfarth H, Katz J, Falck-Ytter Y, Singh S. American Gastroenterological Association Institute technical review on the role of therapeutic drug monitoring in the management of inflammatory bowel diseases. *Gastroenterology* 2017; **153:** 835–57.e6.

18 Cornillie F, Hanauer SB, Diamond RH, et al. Postinduction serum infliximab trough level and decrease of C-reactive protein level are associated with durable sustained response to infliximab: a retrospective analysis of the ACCENT I trial. *Gut* 2014; **63:** 1721–27.

19 Vande Casteele N, Ferrante M, Van Assche G, et al. Trough concentrations of infliximab guide dosing for patients with inflammatory bowel disease. *Gastroenterology* 2015; **148:** 1320–29.e3.

20 D'Haens G, Vermeire S, Lambrecht G, et al. Increasing infliximab dose based on symptoms, biomarkers, and serum drug concentrations does not increase clinical, endoscopic, and corticosteroid-free remission in patients with active luminal Crohn's disease. *Gastroenterology* 2018; **154:** 1343–51.e1.

21 Van Stappen T, Bollen L, Vande Casteele N, et al. Rapid test for infliximab drug concentration allows immediate dose adaptation. *Clin Transl Gastroenterol* 2016; **7:** e206.

22 Pradeu T, Jaeger S, Vivier E. The speed of change: towards a discontinuity theory of immunity? *Nat Rev Immunol* 2013; **13:** 764–69.

23 Ungar B, Engel T, Yablecovitch D, et al. Prospective observational evaluation of time-dependency of adalimumab immunogenicity and drug concentrations: the Poetic study. *Am J Gastroenterol* 2018; **113:** 890–98.

24 Yarur AJ, Kubiliun MJ, Czul F, et al. Concentrations of 6-thioguanine nucleotide correlate with trough levels of infliximab in patients with inflammatory bowel disease on combination therapy. *Clin Gastroenterol Hepatol* 2015; **13:** 1118–24.e3.

25 Roblin X, Boschetti G, Williet N, et al. Azathioprine dose reduction in inflammatory bowel disease patients on combination therapy: an open-label, prospective and randomised clinical trial. *Aliment Pharmacol Ther* 2017; **46:** 142–49.

26 Colombel J-F, Reinisch W, Mantzaris GJ, et al. Randomised clinical trial: deep remission in biologic and immunomodulator naïve patients with Crohn's disease—a SONIC post hoc analysis. *Aliment Pharmacol Ther* 2015; **41:** 734–46.

27 Chalhoub JM, Rimmani HH, Gumaste VV, Sharara AI. Systematic review and meta-analysis: adalimumab monotherapy versus combination therapy with immunomodulators for induction and maintenance of remission and response in patients with Crohn's disease. *Inflamm Bowel Dis* 2017; **23:** 1316–27.

28 Singh S, Facciorusso A, Singh AG, et al. Obesity and response to anti-tumor necrosis factor-α agents in patients with select immune-mediated inflammatory diseases: a systematic review and meta-analysis. *PLoS One* 2018; **13:** e0195123.

29 Juillerat P, Sokol H, Froehlich F, et al. Factors associated with durable response to infliximab in Crohn's disease 5 years and beyond: a multicenter international cohort. *Inflamm Bowel Dis* 2015; **21:** 60–70.

30 Magro F, Rodrigues-Pinto E, Santos-Antunes J, et al. High C-reactive protein in Crohn's disease patients predicts nonresponse to infliximab treatment. *J Crohns Colitis* 2014; **8:** 129–36.

31 Fasanmade AA, Adedokun OJ, Olson A, Strauss R, Davis HM. Serum albumin concentration: a predictive factor of infliximab pharmacokinetics and clinical response in patients with ulcerative colitis. *Int J Clin Pharmacol Ther* 2010; **48**: 297–308.

32 Kirchgesner J, Lemaitre M, Carrat F, Zureik M, Carbonnel F, Dray-Spira R. Risk of serious and opportunistic infections associated with treatment of inflammatory bowel diseases. *Gastroenterology* 2018; **155:** 337–46.e10.

Supplementary appendix: 'Predictors of anti-TNF treatment failure: a prospective multi-centre study of biologic naïve patients with active luminal Crohn's disease.

Table of Contents

IBD Pharmacogenetics Study Group

All UK gastroenterologists were invited to participate in the PANTS study which was promoted through the UK National Institute for Health Service Research (NIHR) and the British Society of Gastroenterology (BSG).

Hospital or Trust name	City	Name	Job Title
Tameside Hospital NHS Foundation Trust	Ashton U Lyne	Dr Vinod Patel	Consultant Gastroenterologist
Basildon and Thurrock University Hospitals NHS Foundation Trust	Basildon	Dr Zia Mazhar	Consultant Gastroenterologist
Hampshire Hospitals NHS Foundation Trust	Basingstoke	Dr Rebecca Saich	Consultant Gastroenterologist
Royal United Hospital	Bath	Dr Ben Colleypriest	Consultant Gastroenterologist
Ulster Hospital	Belfast	Dr Tony C Tham	Consultant Gastroenterologist
University Hospital's Birmingham NHS Foundation Trust	Birmingham	Dr Tariq H Iqbal	Consultant Gastroenterologist
East Lancashire NHS Teaching Trust	Blackburn	Dr Vishal Kaushik	Consultant Gastroenterologist
Blackpool Teaching Hospitals NHS Foundation Trust	Blackpool	Dr Senthil Murugesan	Consultant Gastroenterologist
Bolton NHS Trust	Bolton	Dr Salil Singh	Consultant Gastroenterologist
Royal Bournemouth Hospital	Bournemouth	Dr Sean Weaver	Consultant Gastroenterologist
Bradford Teaching Hospitals Foundation Trust - (St Lukes Hospital &Bradford Royal Infirmary)	Bradford	Dr Cathryn Preston	Consultant Gastroenterologist
Brighton and Sussex University Hospitals NHS Trust	Brighton	Dr Assad Butt	Paediatric Consultant Gastroenterologist
Brighton and Sussex University Hospitals NHS Trust	Brighton	Dr Melissa Smith	Consultant Gastroenterologist
University Hospitals Bristol NHS Foundation Trust	Bristol	Dr Dharamveer Basude	Consultant Paediatric Gastroenterologist
University Hospitals Bristol NHS Foundation Trust	Bristol	Dr Amanda Beale	Consultant Gastroenterologist
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Frimley Park Hospital NHS Foundation Trust	Camberley	Dr Natalie Direkze	Consultant gastroenterologist
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Cambridge University Hospitals NHS Foundation Trust	Cambridge	Dr Franco Torrente	Consultant Paediatric Gastroenterologist
Cambridge University Hospitals NHS Foundation Trust	Cambridge	Dr Juan De La Revella Negro	Research fellow
North Cumbria University Hospitals NHS Trust	Carlisle	Dr Chris Ewen MacDonald	Consultant Gastroenterologist
Ashford & St Peter's Hospitals NHS Foundation Trust	Chertsey	Dr Stephen M Evans	Consultant Gastroenterologist
St Peter's Hospital	Chertsey	Dr Anton V J Gunasekera	Consultant Gastroenterologist
Ashford & St Peter's Hospitals NHS Foundation Trust	Chertsey	Dr Alka Thakur	Paediatric Consultant
Chesterfield Royal NHS Foundation Trust	Chesterfield	Dr David Elphick	Consultant Gastroenterologist
Colchester Hospital University NHS Foundation Trust	Colchester	Dr Achuth Shenoy	Consultant Gastroenterologist
University Hospitals Coventry and Warwickshire NHS Trust	Coventry	Prof Chuka U Nwokolo	Consultant Gastroenterologist
County Durham and Darlington NHS Foundation Trust	Darlington	Dr Anjan Dhar	Consultant Gastroenterologist & Hon. Clinical Lecturer
Derby Hospital NHS Foundation NHS Trust	Derby	Dr Andrew T Cole	Consultant Gastroenterologist
Doncaster and Bassetlaw Hospitals NHS Foundation Trust	Doncaster	Dr Anurag Agrawal	Consultant Gastroenterologist
Dorset County Hospital NHS Foundation Trust	Dorchester	Dr Stephen Bridger	Consultant Gastroenterologist
Dorset County Hospitals Foundation Trust	Dorchester	Dr Julie Doherty	Paediatric Consultant
Dudley Group NHS Foundation Trust	Dudley	Dr Sheldon C Cooper	Consultant Gastroenterologist
Russells Hall Hospital, The Dudley Group NHS Foundation Trust	Dudley	Dr Shanika de Silva	Consultant Gastroenterologist
Ninewells Hospital & Medical School	Dundee	Dr Craig Mowat	Consultant Gastroenterologist
East Sussex Healthcare Trust	Eastborne	Dr Phillip Mayhead	Consultant Gastroenterologist
NHS Lothian	Edinburgh	Dr Charlie Lees	Consultant Gastroenterologist and Honorary Senior Lecturer

Hospital or Trust name	City	Name	Job Title
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Glasgow Royal Infirmary	Glasgow	Dr Daniel R Gaya	Consultant Gastroenterologist
Royal Hospital for Children	Glasgow	Dr Richard K Russell	Consultant Paediatric Gastroenterologist
Royal Hospital for Children	Glasgow	Dr Lisa Gervais	Research fellow
Gloucestershire Hospitals NHS Trust	Gloucester	Dr Paul Dunckley	Consultant Gastroneterologist
United Lincolnshire Hospitals NHS Trust	Grantham	Dr Tariq Mahmood	Consultant Gastroenterologist
James Paget University Hospitals NHS Foundation Trust	Great Yarmouth	Dr Paul J R Banim	Consultant Gastroneterologist
Calderdale and Huddersfield NHS Trust	Halifax	Dr Sunil Sonwalkar	Consultant Gastroenterologist
Princess Alexandra Hospital NHS Trust	Harlow	Dr Deb Ghosh	Consultant Gastroenterologist
Princess Alexandra Hospital NHS Trust	Harlow	Dr Rosemary H Phillips	Consultant Gastroenterologist
Hull and East Yorkshire NHS Trust	Hull	Dr Amer Azaz	Paediatric Consultant Gastroenterologist
Hull and East Yorkshire NHS Trust	Hull	Dr Shaji Sebastian	Consultant Gastroenterologist
Airedale NHS Foundation Trust	Keighley	Dr Richard Shenderey	Consultant Gastroenterologist
Crosshouse Hospital	Kilmarnock	Dr Lawrence Armstrong	Consultant Paediatrician
Crosshouse Hospital	Kilmarnock	Dr Claire Bell	Research fellow
The Queen Elizabeth Hospital NHS Foundation Trust	Kings Lynn	Dr Radhakrishnan Hariraj	Consultant Gastroenterologist
Kingston Hospital NHS Trust	Kingston upon Thames	Dr Helen Matthews	Consultant Gastroenterologist
NHS Fife	Kirkcaldy	Dr Hasnain Jafferbhoy	Consultant Gastroenterologist
Leeds Teaching Hospitals NHS Trust	Leeds	Dr Christian P Selinger	Consultant Gastroenterologist
Leeds Teaching Hospitals NHS Trust	Leeds	Dr Veena Zamvar	Paediatric Consultant Gastroenteorlogist
University Hospitals of Leicester NHS Trust	Leicester	Prof John S De Caestecker	Consultant Gastroenterologist
University Hospitals of Leicester NHS Trust	Leicester	Dr Anne Willmott	Paediatric Consultant Gastroenterologist
Mid Cheshire Hospitals NHS Foundation Trust	Leighton	Mr Richard Miller	Research Nurse
United Lincolnshire Hospitals NHS Trust	Lincoln	Dr Palani Sathish Babu	Consultant Gastroenterologist
Alder Hey Childrens Hospital	Liverpool	Dr Christos Tzivinikos	Consultant Paediatric Gastroenterologist
University College London Hospitals NHS Foundation Trust	London	Dr Stuart L Bloom	Consultant Gastroenterologist
Kings College Hospital NHS Foundation Trust	London	Dr Guy Chung-Faye	Consultant Gastroenterologist
Royal London Childrens Hospital, Barts Health NHS Trust	London	Prof Nicholas M Croft	Paediatric Consultant Gastroenterologist
Chelsea & Westminster Hospital	London	Dr John ME Fell	Consultant Paediatric Gastroenterologist
Chelsea and Westminster Hospital NHS Foundation	London	Dr Marcus Harbord	Consultant Gastroenterologist
North West London Hospitals NHS Trust	London	Dr Ailsa Hart	Consultant Gastroenterologist
Kings College Hospital NHS Foundation Trust	London	Dr Ben Hope	Consultant Paediatrician
Guys & St Thomas' NHS Foundation Trust	London	Dr Peter M Irving	Consultant Gastroenterologist
Barts and The London NHS Trust	London	Prof James O Lindsay	Consultant Gastroenterologist
Guy's and St Thomas' NHS trust	London	Dr Joel E Mawdsley	Gastroenterology Consultant
Lewisham and Greenwich Healthcare NHS Trust	London	Dr Alistair McNair	Consultant Gastroenterologist
Chelsea and Westminster Hospital NHS Foundation	London	Dr Kevin J Monahan	Consultant Gastroenterologist
Royal Free London NHS Foundation Trust	London	Dr Charles D Murray	Consultant Gastroenterologist
Imperial College Healthcare NHS Trust	London	Prof Timothy Orchard	Consultant Gastroenterologist
St George's Healthcare NHS Trust	London	Dr Thankam Paul	Paediatric Consultant Gastroenterologist
St George's Healthcare NHS Trust	London	Dr Richard Pollok	Reader and Consultant Gastroenterologist
Great Ormond Street Hospital for Children NHS Foundation Trust	London	Dr Neil Shah	Consultant Gastroenterologist

Hospital or Trust name	City	Name	Job Title
North West London Hospitals NHS Trust	London	Dr Sonia Bouri	Research fellow
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Luton and Dunstable Hospital Foundation Trust	Luton	Dr Anita Modi	Paediatric Consultant with Allergy and Gastroenterology interest
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Maidstone and Tunbridge Wells NHS Trust	Maidstone	Prof Bim Bhaduri	Paediatric Consultant Gastroenterologist
Manchester University Hospitals NHS Foundation Trust	Manchester	Dr Andrew Adebayo Fagbemi	Consultant Gastroenterologist
Central Manchester University Hospitals NHS Foundation Trust	Manchester	Dr Scott Levison	Consultant Gastroenterologist
The Pennine Acute Hospitals NHS Trust	Manchester	Dr Jimmy K Limdi	Consultant Gastroenterologist
Manchester University NHS Foundation Trust, Wythenshawe Hospital	Manchester	Dr Gill Watts	Consultant Gastroenterologist
Sherwood Forest Hospitals NHS Foundation Trust	Mansfield	Dr Stephen Foley	Consultant Gastroenterologist
South Tees Hospital NHS Foundation Trust	Middlesbrough	Dr Arvind Ramadas	Consultant Gastroenterologist
Milton Keynes Hospital NHS Foundation Trust	Milton Keynes	Dr George MacFaul	Consultant Gastroenterologist
Newcastle Upon Tyne Hospital Trust	Newcastle	Dr John Mansfield	Consultant Gastroenterologist
Isle of Wight NHS Foundation Trust	Newport	Dr Leonie Grellier	Consultant Gastroenterologist
Norfolk & Norwich University Hospital NHS Foundation Trust	Norwich	Dr Mary-Anne Morris	Consultant Paediatric Gastroenterologist
Norfolk & Norwich University Hospital NHS Foundation Trust	Norwich	Dr Mark Tremelling	Consultant Gastroenterologist
Nottingham University Hospitals NHS Trust	Nottingham	Prof Chris Hawkey	Consultant Gastroenterologist
Nottingham University Hospitals NHS Trust	Nottingham	Dr Sian Kirkham	Consultant Paediatric Gastroenterologist
Nottingham University Hospitals NHS Trust	Nottingham	Dr Charles PJ Charlton	Consultant gastroenterologist
Oxford University Hospitals NHS Foundation Trust	Oxford	Dr Astor Rodrigues	Paediatric Consultant Gastroenterologist
Oxford University Hospitals NHS Trust	Oxford	Prof Alison Simmons	Consultant Gastroenterologist
Plymouth Hospitals NHS Trust	Plymouth	Dr Stephen J Lewis	Consultant Gastroenterologist
Poole Hospital NHS Foundation Trust	Poole	Dr Jonathon Snook	Consultant Gastroenterologist
Poole Hospital NHS Foundation Trust	Poole	Dr Mark Tighe	Paediatric Consultant with interest in Oncology and Gastroenterology
Portsmouth Hospitals NHS Trust	Portsmouth	Dr Patrick M Goggin	Consultant Gastroenterologist
Royal Berkshire NHS Foundation Trust	Reading	Dr Aminda N De Silva	Consultant Gastroenterologist
Salford Royal NHS Foundation Trust	Salford	Prof Simon Lal	Consultant Gastroenterologist
Shrewsbury and Telford Hospital NHS Trust	Shrewsbury	Dr Mark S Smith	Consultant Gastroenterologist
South Tyneside NHS Foundation Trust	South Shields	Dr Simon Panter	Consultant Gastroenterologist
Southampton University Hospitals NHS Trust	Southampton	Dr Fraser Cummings	Consultant Gastroenterologist
Southampton University Hospitals NHS Trust	Southampton	Dr Suranga Dharmisari	Research fellow
East and North Herts NHS Trust	Stevenage	Dr Martyn Carter	Consultant Gastroenterologist
NHS Forth Valley	Stirling	Dr David Watts	Consultant Gastroenterologist
Stockport NHS foundation Trust	Stockport	Dr Zahid Mahmood	Consultant Gastroenterologist
North Tees and Hartlepool NHS Foundation Trust	Stockton	Dr Bruce McLain	Paediatric Consultant Gastroenterologist
University Hospitals of North Staffordshire	Stoke-on Trent	Dr Sandip Sen	Consultant Gastroenterologist
University Hospitals of North Midlands NHS Trust	Stoke-on-Trent	Dr Anna J Pigott	Consultant Paediatric Gastroenterologist
City Hospitals Sunderland NHS Foundation Trust	Sunderland	Dr David Hobday	Consultant Gastroenterologist
Taunton and Somerset NHS Foundation Trust	Taunton	Dr Emma Wesley	Consultant Gastroenterologist

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Hospital or Trust name	City	Name	Job Title
South Devon Healthcare NHS Foundation Trust	Torquay	Dr Richard Johnston	Consultant Gastroenterologist
South Devon Healthcare NHS Foundation Trust	Torquay	Dr Cathryn Edwards	Consultant gastroenterologist
Royal Cornwall Hospitals NHS Trust	Truro	Dr John Beckly	Consultant Gastroenterologist
Mid Yorkshire Hospitals NHS Trust	Wakefield	Dr Deven Vani	Consultant Physician & Gastroenterologist
Warrington& Halton NHS Foundation	Warrington	Dr Subramaniam Ramakrishnan	Consultant Gastroenterologist
West Hertfordshire Hospitals NHS Trust	Watford	Dr Rakesh Chaudhary	Consultant Gastroenterologist
Sandwell and West Birmingham Hospitals NHS Trust	West Bromwich	Dr Nigel J Trudgill	Consultant Gastroenterologist
Sandwell and West Birmingham Hospitals NHS Trust	West Bromwich	Dr Rachel Cooney	Consultant gastroenterologist
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Hampshire Hospitals NHS Foundation Trust	Winchester	Dr John N Gordon	Consultant Gastroenterologist
Royal Wolverhampton Hospitals NHS Trust	Wolverhampton	Prof Matthew J Brookes	Consultant Gastroenterologist
Western Sussex Hospitals NHS Trust	Worthing	Dr Andy Li	Consultant Gastroenterologist
Yeovil District Hospital NHS Foundation Trust	Yeovil	Dr Stephen Gore	Consultant Gastroenterologist

Recruitment by site

Centre	Principal Investigator	Original recruits	Taken on from another site
Royal Devon and Exeter NHS Foundation Trust, Exeter	Dr Tariq Ahmad	85	1
Southampton University Hospitals NHS Trust, Southampton	Dr Fraser Cummings	53	
Hull and East Yorkshire NHS Trust, Hull	Dr Shaji Sebastian	42	1
Mid Yorkshire Hospitals NHS Trust, Wakefield	Dr Deven Vani	39	1
Newcastle Upon Tyne Hospital Trust, Newcastle	Dr John Mansfield	40	
Barts and The London NHS Trust, London	Prof James O Lindsay	38	
North West London Hospitals NHS Trust, London	Dr Ailsa Hart	34	
University Hospitals Coventry and Warwickshire NHS Trust, Coventry	Prof Chuka U Nwokolo	34	
Weston Area Health NHS Trust, Weston-Super-Mare	Dr Andy Bell	33	
Leeds Teaching Hospitals NHS Trust, Leeds	Dr Christian P Selinger	31	
Gloucestershire Hospitals NHS Trust, Gloucester	Dr Paul Dunckley	29	
South Devon Healthcare NHS Foundation Trust, Torquay	Dr Richard Johnston	29	
Central Manchester University Hospitals NHS Foundation Trust, Manchester	Dr Scott Levison	27	
Portsmouth Hospitals NHS Trust, Portsmouth	Dr Patrick M Goggin	27	
Hampshire Hospitals NHS Foundation Trust, Winchester	Dr John N Gordon	27	
Poole Hospital NHS Foundation Trust, Poole	Dr Jonathon Snook	26	
Shrewsbury and Telford Hospital NHS Trust, Shrewsbury	Dr Mark S Smith	26	
Sherwood Forest Hospitals NHS Foundation Trust, Mansfield	Dr Stephen Foley	24	
NHS Lothian, Edinburgh	Dr Charlie Lees	24	
Royal Hospital for Children, Glasgow	Dr Richard K Russell	24	
Cambridge University Hospitals NHS Foundation Trust, Cambridge	Dr Miles Parkes	23	
NHS Forth Valley, Stirling	Dr David Watts	23	
Hampshire Hospitals NHS Foundation Trust, Basingstoke	Dr Rebecca Saich	22	
Plymouth Hospitals NHS Trust, Plymouth	Dr Stephen J Lewis	22	
Princess Alexandra Hospital NHS Trust, harlow	Dr Deb Ghosh	22	
Guys & St Thomas' NHS Foundation Trust, London	Dr Peter M Irving	21	
Warrington& Halton NHS Foundation, Warrington	Dr Subramaniam Ramakrishnan	21	
Bradford Teaching Hospitals Foundation Trust - (St Lukes Hospital &Bradford Royal Infirmary), Bradford	Dr Cathryn Preston	19	
Royal Bournemouth Hospital, Bournemouth	Dr Sean Weaver	18	1
Sandwell and West Birmingham Hospitals NHS Trust, West Bromwich	Dr Nigel J Trudgill	19	
Glasgow Royal Infirmary, Glasgow	Dr Daniel R Gaya	18	
St George's Healthcare NHS Trust, London	Dr Richard Pollok	18	
Western Sussex Hospitals NHS Trust	Dr Andy Li	16	2
University Hospitals Bristol NHS Foundation Trust, Bristol	Dr Amanda Beale	15	1
Great Ormond Street Hospital for Children NHS Foundation Trust, London	Dr Neil Shah	16	
Royal Berkshire NHS Foundation Trust, Reading	Dr Aminda N De Silva	15	1
Royal London Childrens Hospital, Barts Health NHS Trust, London	Prof Nicholas M Croft	16	
City Hospitals Sunderland NHS Foundation Trust, Sunderland	Dr David Hobday	16	

Centre	Principal Investigator	Original recruits	Taken on from another site
Blackpool Teaching Hospitals NHS Foundation Trust, Blackpool	Dr Senthil Murugesan	15	
Colchester Hospital University NHS Foundation Trust, Colchester	Dr Achuth Shenoy	15	
Norfolk & Norwich University Hospital NHS Foundation Trust, Norwich	Dr Mark Tremelling	15	
University Hospitals of Leicester NHS Trust, Leicester	Dr Anne Willmott	15	
Manchester University NHS Foundation Trust, Wythenshawe Hospital, Manchester	Dr Gill Watts	15	
Basildon and Thurrock University Hospitals NHS Foundation Trust, Basildon	Dr Zia Mazhar	14	
Derby Hospital NHS Foundation NHS Trust, derby	Dr Andrew T Cole	14	
South Tees Hospital NHS Foundation Trust, Middlesbrough	Dr Arvind Ramadas	14	
East and North Herts NHS Trust, Stevenage	Dr Martyn Carter	14	
South Tyneside NHS Foundation Trust, South Shields	Dr Simon Panter	14	
University Hospital's Birmingham NHS Foundation Trust, Birmingham	Dr Tariq H Iqbal	14	
Taunton and Somerset NHS Foundation Trust, Taunton	Dr Emma Wesley	13	
Tameside Hospital NHS Foundation Trust, Ashton U Lyne	Dr Vinod Patel	13	
Yeovil District Hospital NHS Foundation Trust, Yeovil	Dr Stephen Gore	13	
Dorset County Hospital NHS Foundation Trust, Dorchester	Dr Stephen Bridger	12	
Royal Wolverhampton Hospitals NHS Trust, Wolverhampton	Prof Matthew J Brookes	12	
Nottingham University Hospitals NHS Trust, Nottingham	Dr Sian Kirkham	12	
Airedale NHS Foundation Trust, Keighley	Dr Richard Shenderey	11	
The Queen Elizabeth Hospital NHS Foundation Trust, Kings Lynn	Dr Radhakrishnan Hariraj	11	
Royal Cornwall Hospitals NHS Trust, Truro	Dr John Beckly	11	
Royal United Hospital, Bath	Dr Ben Colleypriest	10	1
University Hospitals of North Midlands Nhs Trust, Stoke-on-Trent	Dr Anna J Pigott	11	
Chesterfield Royal NHS Foundation Trust, Chesterfield	Dr David Elphick	10	
Leeds Teaching Hospitals NHS Trust, Leeds	Dr Veena Zamvar	9	1
Luton and Dunstable Hospital Foundation Trust, Luton	Dr Anita Modi	9	1
Nottingham University Hospitals NHS Trust, Nottingham	Prof Chris Hawkey	10	
Cambridge University Hospitals NHS Foundation Trust, Cambridge	Dr Franco Torrente	9	
The Luton & Dunstable University Hospital, Luton	Dr Matt W Johnson	9	
Royal Albert Edward Infirmary, Wrightington, Wigan & Leigh NHS Foundation Trust, Wigan	Dr Neeraj Prasad	9	
Brighton and Sussex University Hospitals NHS Trust, Brighton	Dr Assad Butt	9	
Russells Hall Hospital, The Dudley Group NHS Foundation Trust, Dudley	Dr Shanika de Silva	9	
Stockport NHS foundation Trust, Stockport	Dr Zahid Mahmood	9	
The Pennine Acute Hospitals NHS Trust, Manchester	Dr Jimmy K Limdi	9	
Calderdale and Huddersfield NHS Trust, Halifax	Dr Sunil Sonwalkar	8	
Chelsea & Westminster Hospital, London	Dr John ME Fell	8	
Milton Keynes Hospital NHS Foundation Trust, Milton Keynes	Dr George MacFaul	8	
James Paget University Hospitals NHS Foundation Trust, Great Yarmouth	Dr Paul J R Banim	6	1

Centre	Principal Investigator	Original recruits	Taken on from another site
Oxford University Hospitals NHS Foundation Trust, Oxford	Dr Astor Rodrigues	7	
Bolton NHS Trust, Bolton	Dr Salil Singh	7	
Brighton and Sussex University Hospitals NHS Trust, Brighton	Dr Melissa Smith	5	2
County Durham and Darlington NHS Foundation Trust, Darlington	Dr Anjan Dhar	5	1
Kings College Hospital NHS Foundation Trust, London	Dr Guy Chung-Faye	5	1
Lewisham and Greenwich Healthcare NHS Trust, London	Dr Alistair McNair	6	
Royal Free London NHS Foundation Trust, London	Dr Charles D Murray	6	
University College London Hospitals NHS Foundation Trust, London	Dr Stuart L Bloom	6	
University Hospitals Bristol NHS Foundation Trust, Bristol	Dr Dharamveer Basude	5	
Doncaster and Bassetlaw Hospitals NHS Foundation Trust, Doncaster	Dr Anurag Agrawal	5	
Frimley Park Hospital NHS Foundation Trust, Camberley	Dr Sarah Langlands	5	
Maidstone and Tunbridge Wells NHS Trust, Maidstone	Dr B K Baburajan	4	1
Manchester University Hospitals NHS Foundation Trust, Manchester	Dr Andrew Adebayo Fagbemi	5	
Ninewells Hospital & Medical School, Dundee	Dr Craig Mowat	5	
Ulster Hospital, Belfast	Dr Tony C Tham	5	
North Tees and Hartlepool NHS Foundation Trust, Stockton	Dr Bruce McLain	5	
West Hertfordshire Hospitals NHS Trust, Watford	Dr Rakesh Chaudhary	5	
Chelsea and Westminster Hospital NHS Foundation, London	Dr Marcus Harbord	4	
East Sussex Healthcare Trust, Eastborne	Dr Phillip Mayhead	4	
Hull and East Yorkshire NHS Trust, Hull	Dr Amer Azaz	4	
Imperial College Healthcare NHS Trust, London	Prof Timothy Orchard	4	
NHS Fife, Kirkcaldy	Dr Hasnain Jafferbhoy	4	
Mid Cheshire Hospitals NHS Foundation Trust, NA	Mr Richard Miller	4	
United Lincolnshire Hospitals NHS Trust, Lincoln	Dr Palani Sathish Babu	4	
Norfolk & Norwich University Hospital NHS Foundation Trust, Norwich	Dr Mary-Anne Morris	4	
Alder Hey Childrens Hospital, Liverpool	Dr Christos Tzivinikos	3	
Crosshouse Hospital, Kilmarnock	Dr Lawrence Armstrong		3
North Cumbria University Hospitals NHS Trust, NA	Dr Chris MacDonald	3	
United Lincolnshire Hospitals NHS Trust, grantham	Dr Tariq Mahmood	3	
East Lancashire NHS Teaching Trust, Blackburn	Dr Vishal Kaushik	3	
Salford Royal NHS Foundation Trust, Salford	Prof Simon Lal	3	
University Hospitals of North Staffordshire, Stoke-on Trent	Dr Sandip Sen	3	
Chelsea and Westminster Hospital NHS Foundation, London	Dr Kevin J Monahan	3	
University Hospitals of Leicester NHS Trust, Leicester	Prof John S De Caestecker	2	
Oxford University Hospitals NHS Trust, Oxford	Prof Alison Simmons	1	1
Poole Hospital NHS Foundation Trust, Poole	Dr Mark Tighe	2	
Ashford & St Peter's Hospitals NHS Foundation Trust, Chertsey	Dr Stephen M Evans	2	
North Cumbria University Hospitals NHS Trust, Carlisle	Dr Chris Ewen MacDonald	2	

Centre	Principal Investigator	Original recruits	Taken on from another site
Dorset County Hospitals Foundation Trust, Dorchester	Dr Julie Doherty	1	
Kings College Hospital NHS Foundation Trust, London	Dr Ben Hope	1	
Kingston Hospital NHS Trust, Kingston upon Thames	Dr Helen Matthews	1	
Maidstone and Tunbridge Wells NHS Trust, NA	Prof Bim Bhaduri	1	
St George's Healthcare NHS Trust, London	Dr Thankam Paul	1	
Isle of Wight NHS Foundation Trust, Newport	Dr Leonie Grellier	1	
Ashford & St Peter's Hospitals NHS Foundation Trust, Chertsey	Dr Alka Thakur		1

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Supplementary methods

Laboratory assay information

Drug and antibody levels

Serum infliximab and adalimumab drug levels were analyzed on the Dynex (Chantilly Virginia, USA) DS2 automated Enzyme-Linked ImmunoSorbent Assay (ELISA) platform, using the Immundiagnostik IDKmonitor infliximab and adalimumab drug level ELISA assays. These assays allow quantitative determination of free infliximab and adalimumab using a sandwich ELISA technique. The IDK monitor infliximab drug level assay has a measuring range of 0·8-45mg/L, with an intra-assay CV of <9·7% and an inter-assay CV of <11·0%. The IDK monitor adalimumab drug level assay has a measuring range of 0·8-45mg/L, with an intra-assay CV of <2·6% and an inter-assay CV of <13·0%.

Serum infliximab and adalimumab total anti-drug antibody levels were analysed on the Dynex DS2 automated ELISA platform using the IDK monitor infliximab & adalimumab total anti-drug-antibody ELISA assays. These assays allow the detection of total antibodies against infliximab; measuring free and bound antibodies against infliximab. The IDK monitor Infliximab total ADA assay has a measuring range of 10-400 AU/mL, with an intra-assay CV of <4·7% and an inter-assay CV of <12·6%. The IDK monitor adalimumab drug level assay has a measuring range of 10-200 AU/mL, with an intra-assay CV of <7·2% and an inter-assay CV of <5·9%. All assays including drug and antibody levels were tested for stability.>

C-reactive protein (CRP)

Serum CRP analysis was carried out on the 702 module of the Roche Diagnostics Cobas 8000 analyzer using the Cobas Generation 3 CRP assay. This assay quantitative determination of CRP using a particle-enhanced immunoturbidimetric assay. The measuring range of this assay is 0·3-350mg/L with an intra-assay CV <3·7% and an interassay precision <4%.

Supplementary reference

1 Perry M, Bewshea C, Brown R, So K, Ahmad T, McDonald T. Infliximab and adalimumab are stable in whole blood clotted samples for seven days at room temperature. *Ann Clin Biochem* 2015; **52**: 672–4.

Supplementary figures

Figure S1: Schedule of visit timing for patients in the PANTS study

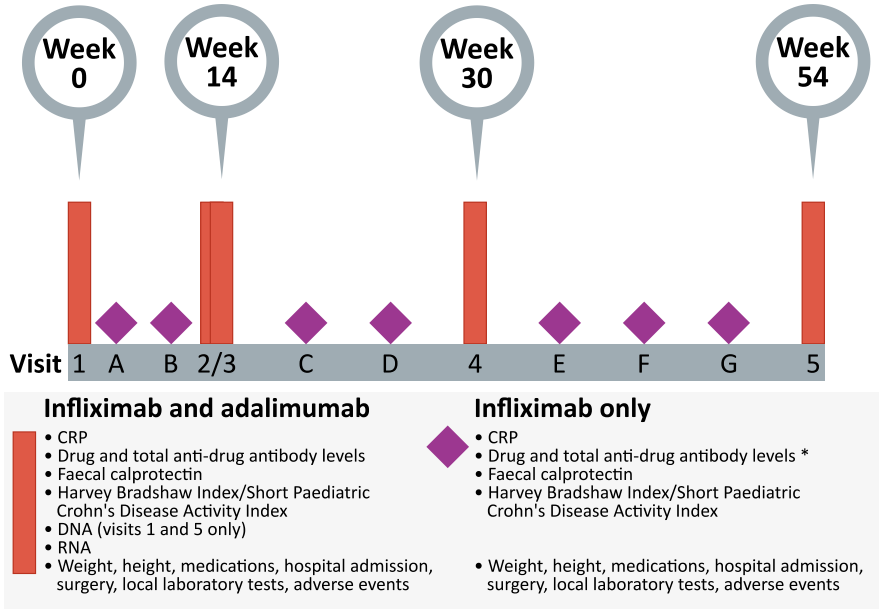

Figure S2: Study timing for PANTS showing distribution of actual visit timings and the windows used to include visits as vertical lines

Visit	Minimum	5th centile	25th centile (lower quartile)	Median	75th centile (upper quartile)	95th centile	Maximum
Week 0	-4·0	-0·9	0·0	0·0	0·0	0·0	0·0
Week 14	10·0	11·6	13·2	14·0	14·3	16·4	20·0
Week 54	42·0	46·9	52·7	54·0	55·4	59·1	66·0

Figure S3: Definitions of outcomes at week 14

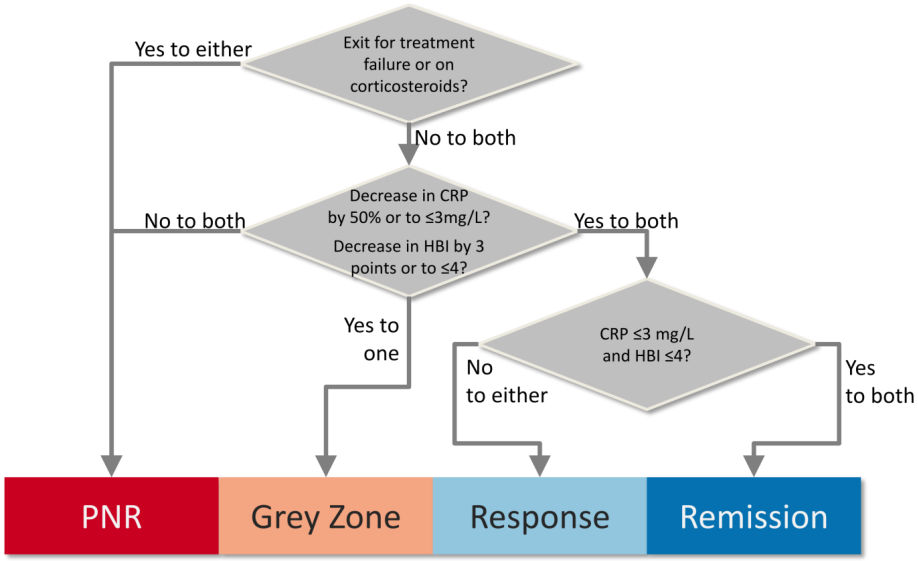

Figure S4: Survival curves of loss of response or exit among patients who did not have primary non-response or exit prior to week 14 stratified by anti-TNF drug

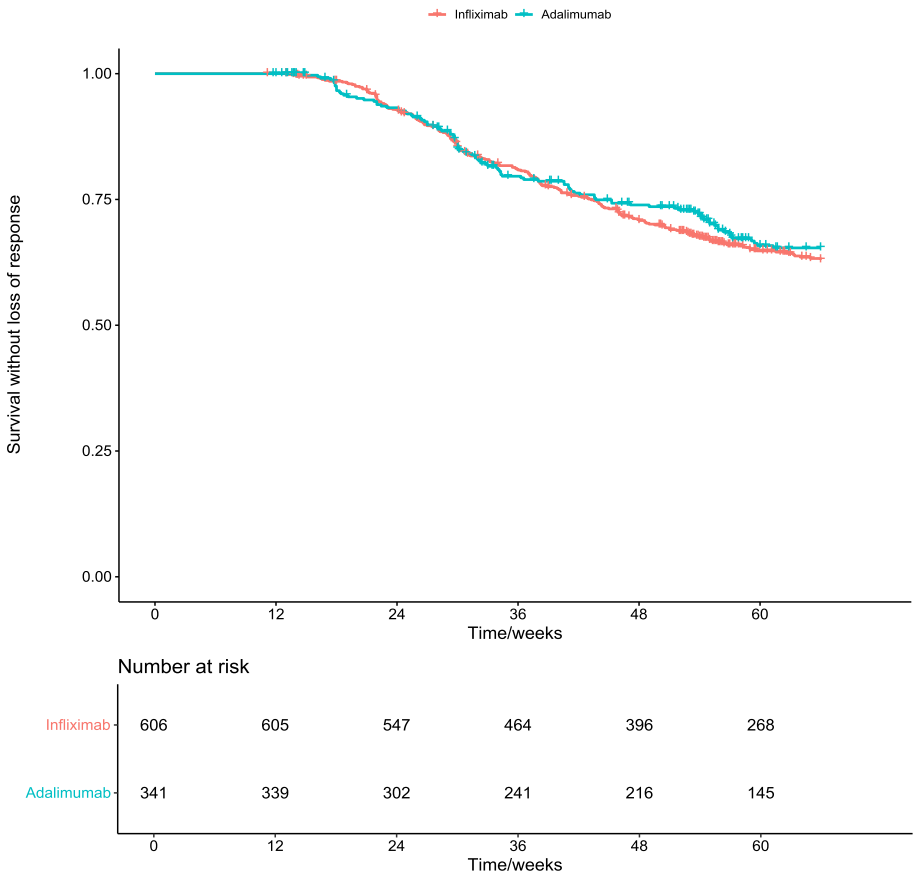

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Figure S5: Survival curves of loss of response or exit among patients who did not have primary non-response or exit prior to week 14 – Remicade vs. CTP-13

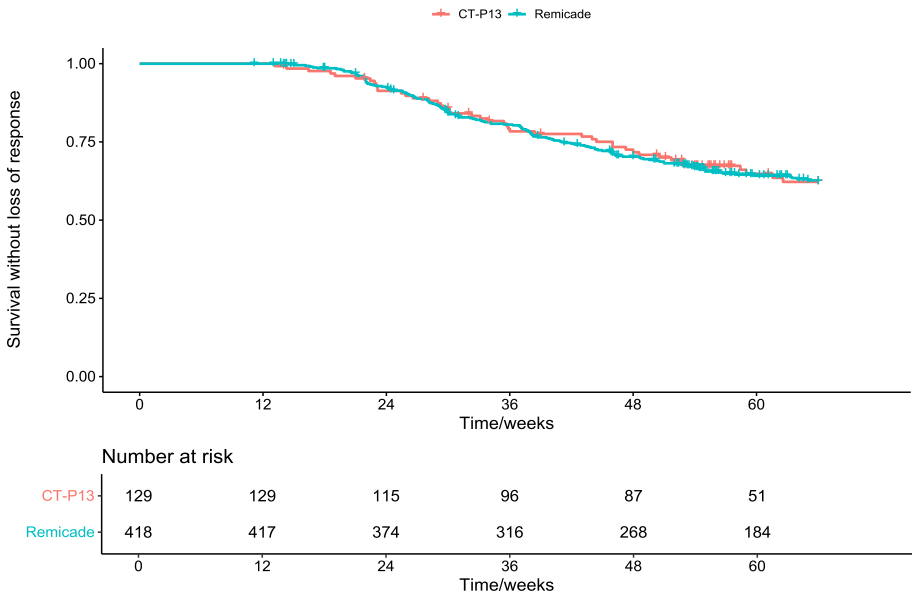
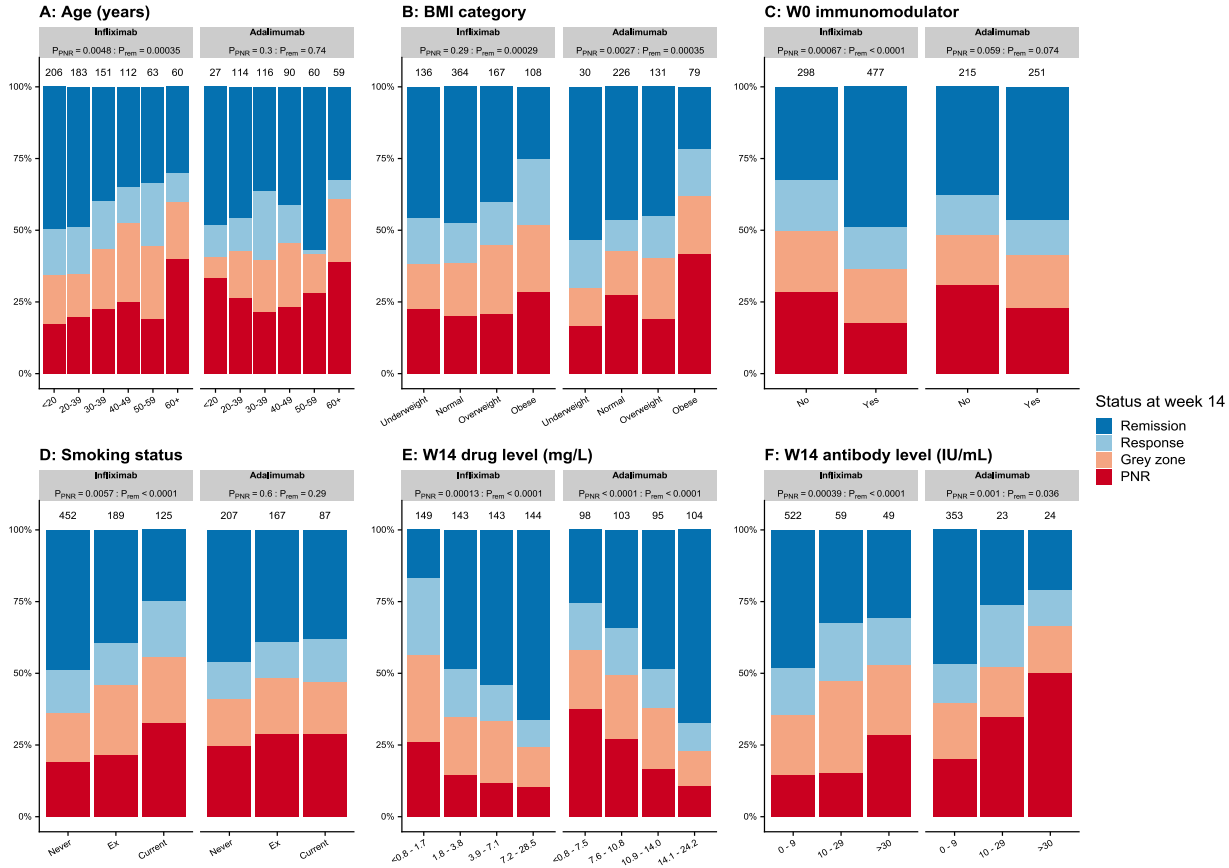


Figure S6: Univariable associations with outcome at week 14 stratified by drug type



P values shown represent Fisher's exact test for categorical variables and Mann Whitney U for continuous variables (drug and antibody levels), although for graphical purposes these have been split into predefined groups (age, antibody) or quartiles (drug level). Abbreviations: BMI: body mass index; PNR: primary non-response; w: week

Figure S7: Optimal week 14 drug levels for infliximab and adalimumab

A. Week 14 drug level and clinical status at week 14; B. Week 14 drug level and clinical status at week 54

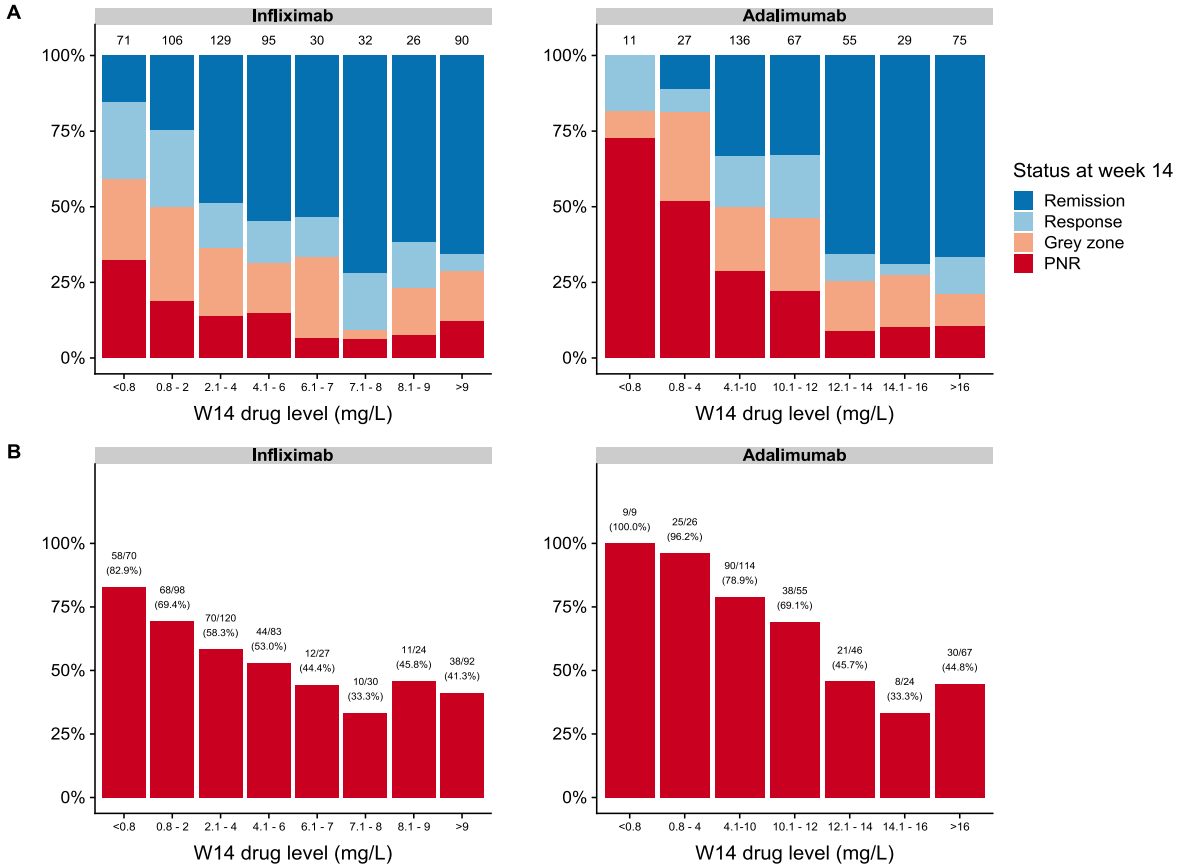


Figure S8: Optimal week 6 drug levels for infliximab

A. Week 6 drug level and clinical status at week 14; B. Week 6 drug level and clinical status at week 54

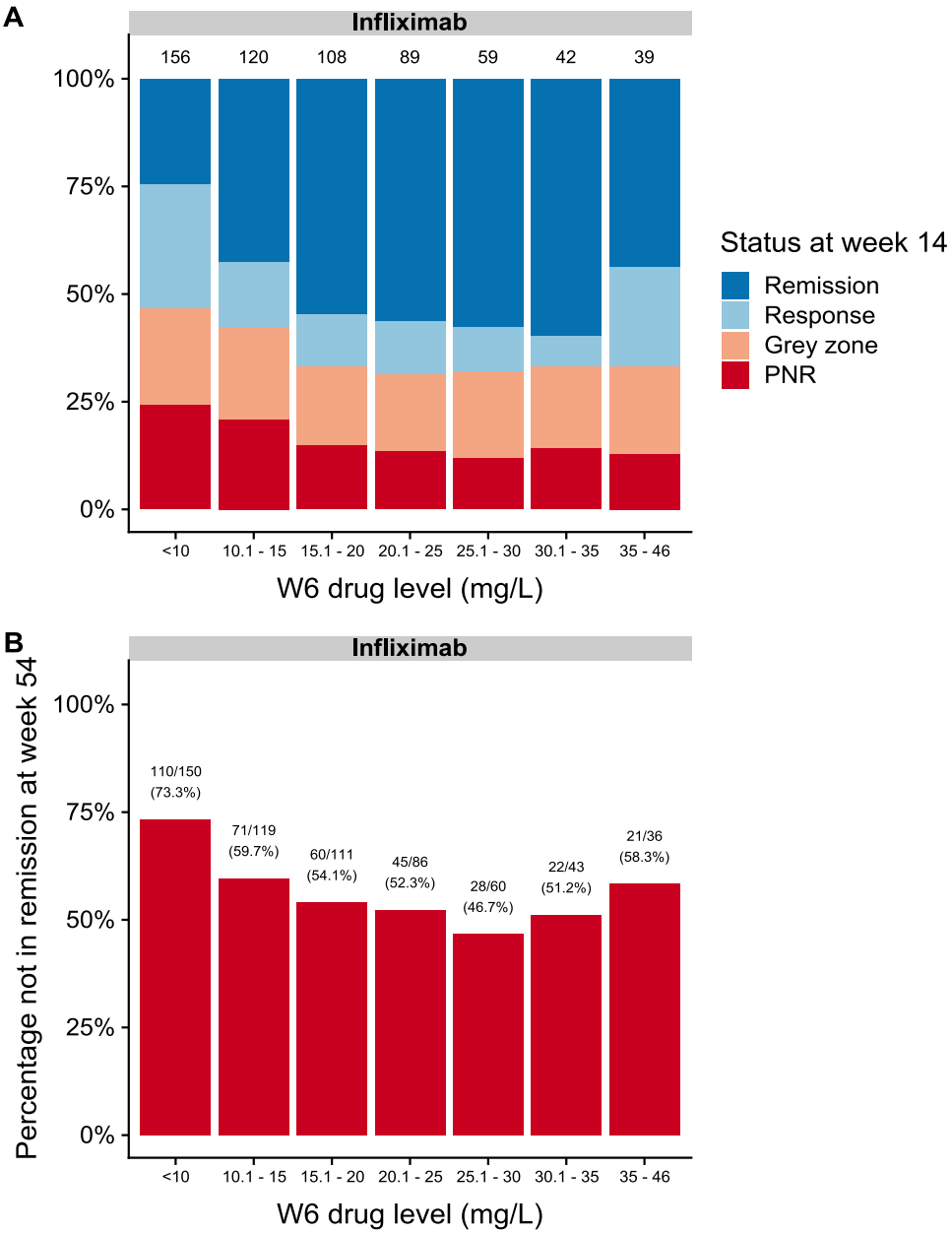
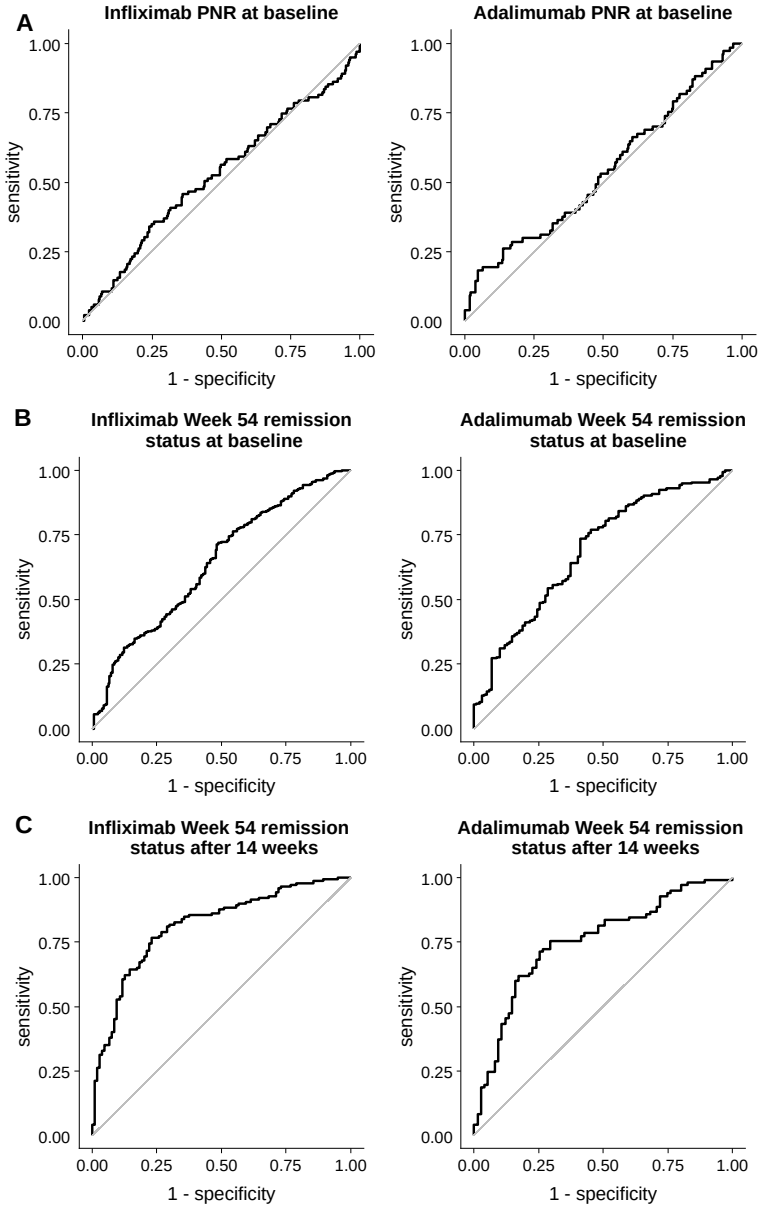


Figure S9: ROC Curves for Logistic Regression Models

A ROC curves for the predictive model of PNR to infliximab (left) and adalimumab (right) with optimal probability threshold for prediction (defined by maximum of specificity and sensitivity)

B ROC curves for the predictive model of week 54 non-remission to infliximab (left) and adalimumab (right) from baseline data with optimal probability threshold for prediction (defined by maximum of specificity and sensitivity)

C ROC curves for the predictive model of week 54 non-remission to infliximab (left) and adalimumab (right) from week 14 data with optimal probability threshold for prediction (defined by maximum of specificity and sensitivity)



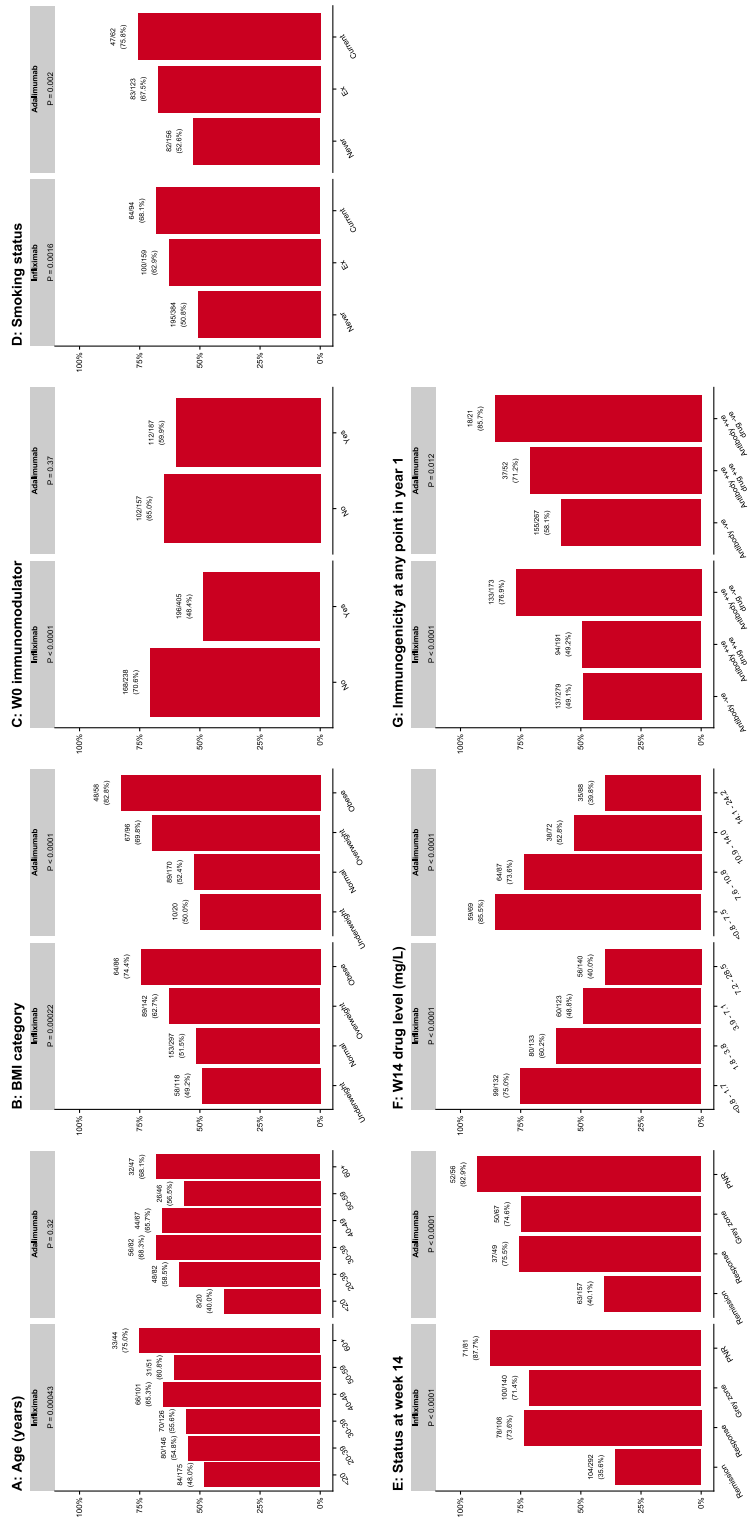
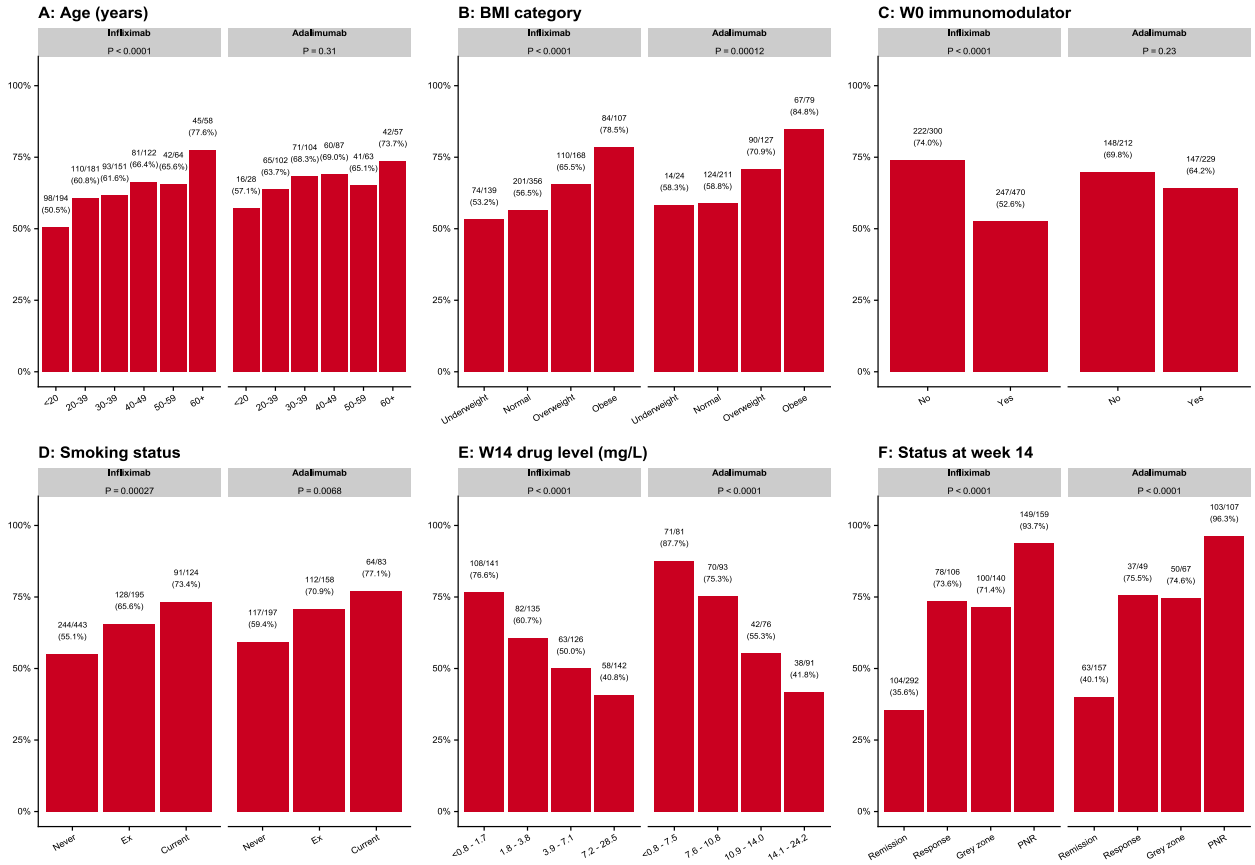


Figure S10: Univariable associations with non-remission at week 54, excluding patients who exited for primary non-response and stratified by drug type
P values shown represent Fisher's exact test for categorical variables and Mann Whitney U for continuous variables (drug and antibody levels), although for graphical purposes these have been split into predefined groups (age, antibody) or quartiles (drug level). Abbreviations: BMI: body mass index; w: week

Figure S11: Univariable analyses of non-remission at week 54 (all patients including those with exit prior to week 14)



P values shown are for Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables, although for graphical purposes these have been split into predefined groups (age) or quartiles (drug level). Abbreviations: BMI – body mass index, W – week.

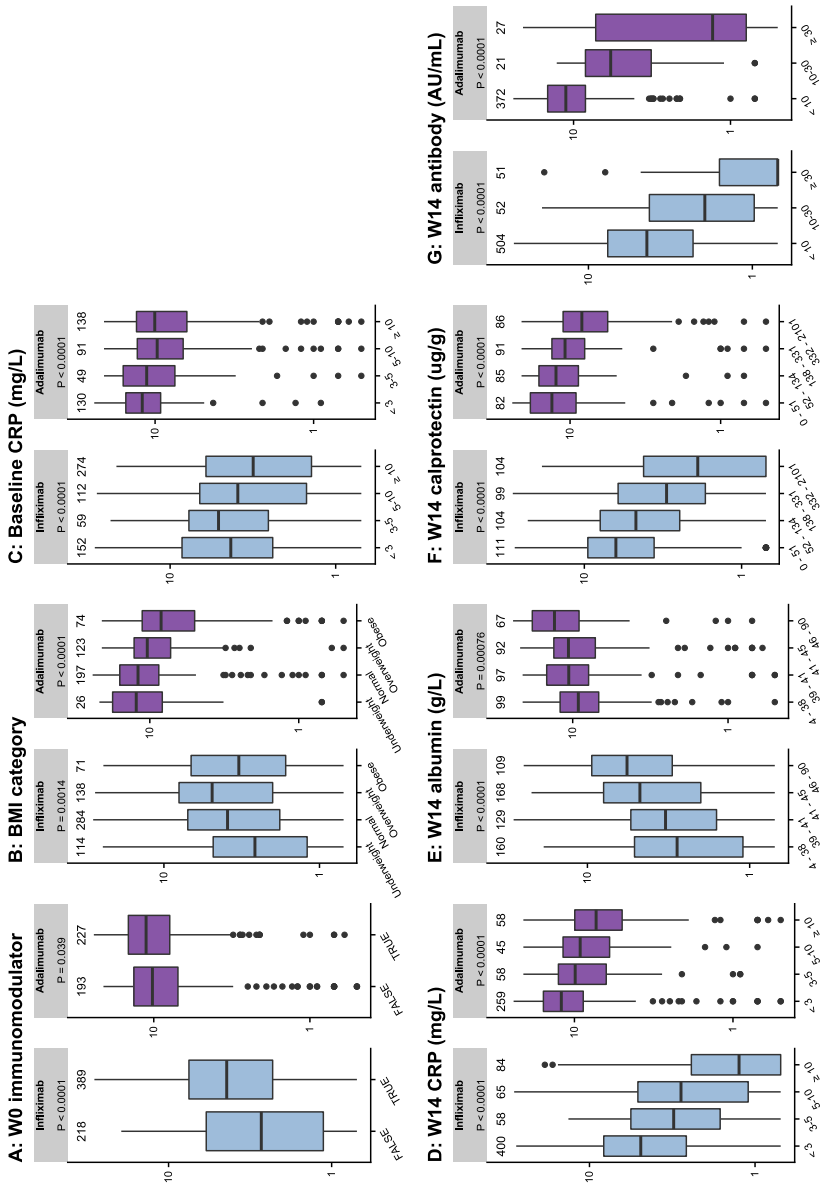
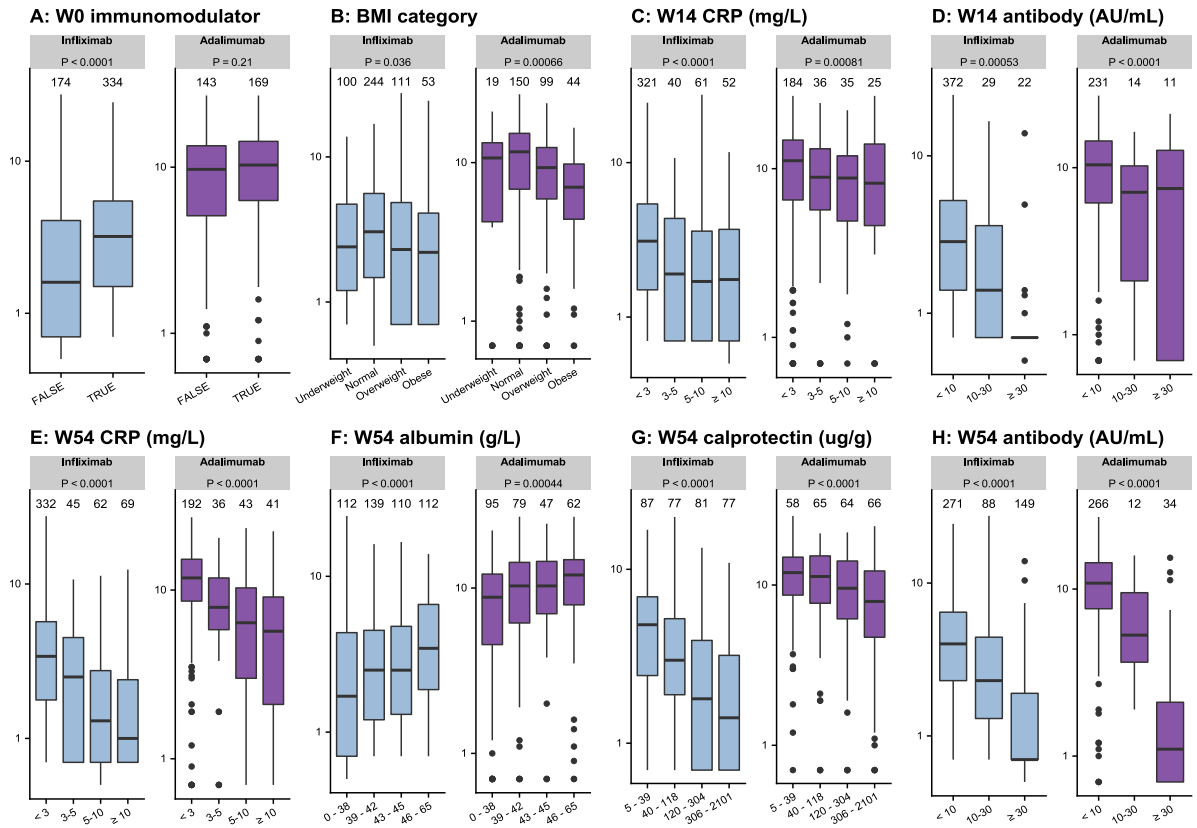


Figure S12: Univariable associations with drug level at week 14, stratified by drug type

Number shown above each plot is number per group. P values shown represent Mann Whitney U or Kruskal Wallis tests for categorical variables (drug and antibody levels) and Spearman's rank correlation tests for continuous variables, although these have been split into predefined groups (CRP, antibody) or quartiles (albumin, calprotectin) for graphical purposes. Abbreviations: w: week.

Figure S13: Univariable associations with drug level at week 54, stratified by drug type



Number shown above each plot is number per group. P values shown represent Mann Whitney U or Kruskal Wallis tests for categorical variables (drug and antibody levels) and Spearman's rank correlation tests for continuous variables, although these have been split into predefined groups (CRP, antibody) or quartiles (albumin, calprotectin) for graphical purposes. Abbreviations: w: week.

Figure S14: Evolution of immunogenicity by drug – antibody≥10AU/ml

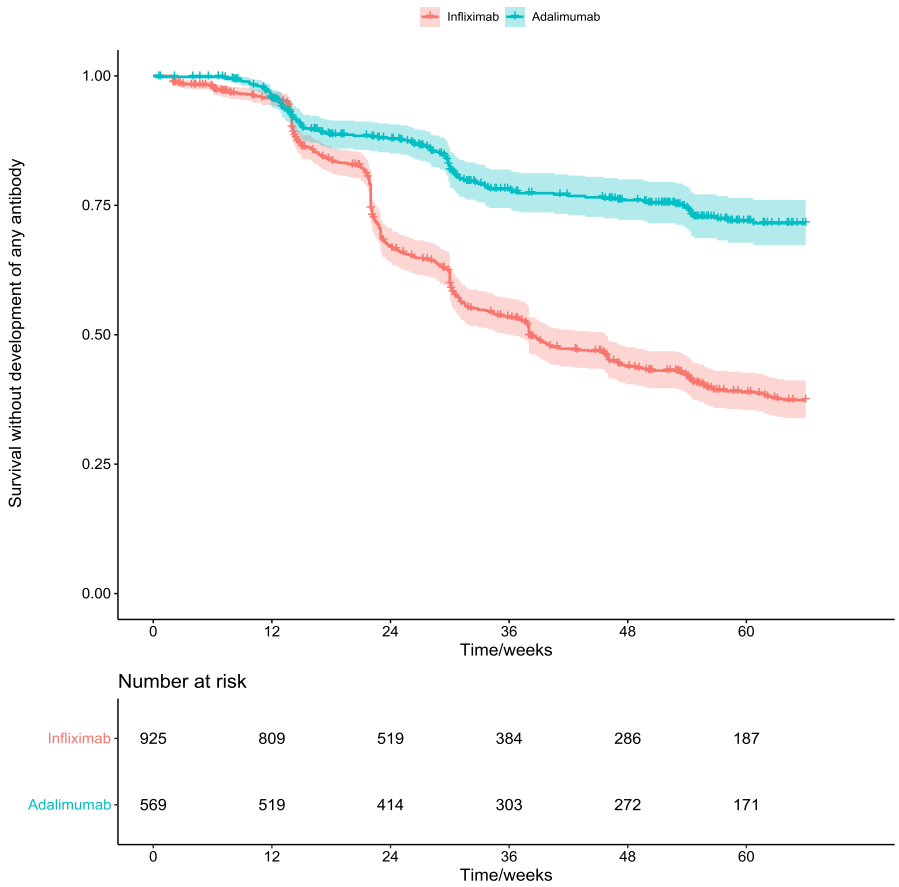


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Figure S15: Evolution of immunogenicity by drug – antibody>10AU/ml. CT-P13 and Remicade separately

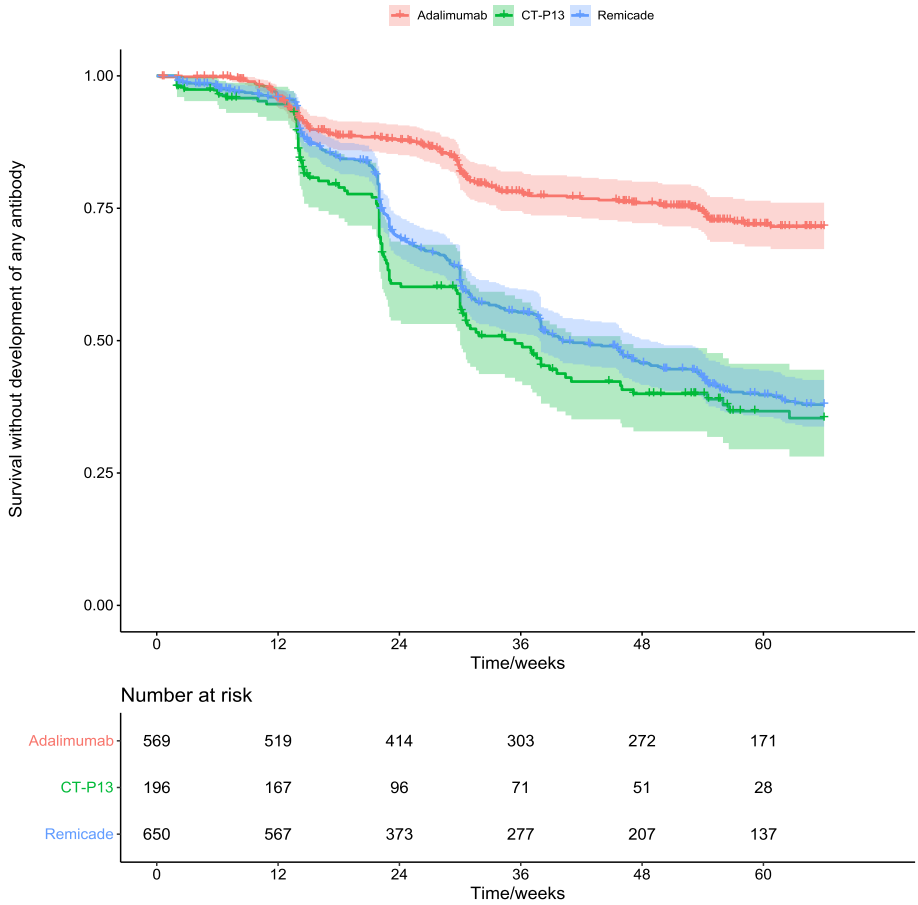

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Figure S16: Evolution of immunogenicity by drug – antibody≥10AU/ml and undetectable drug

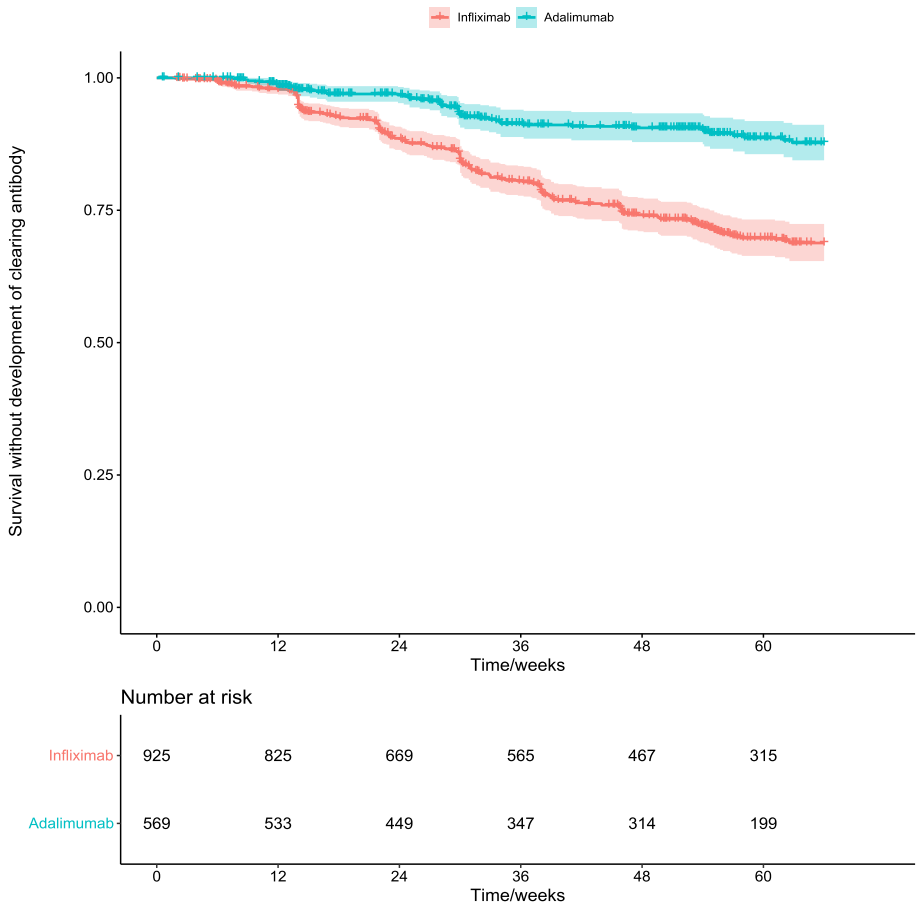

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Figure S17: Evolution of immunogenicity by drug – antibody≥10AU/ml and undetectable drug. CT-P13 and Remicade separately.

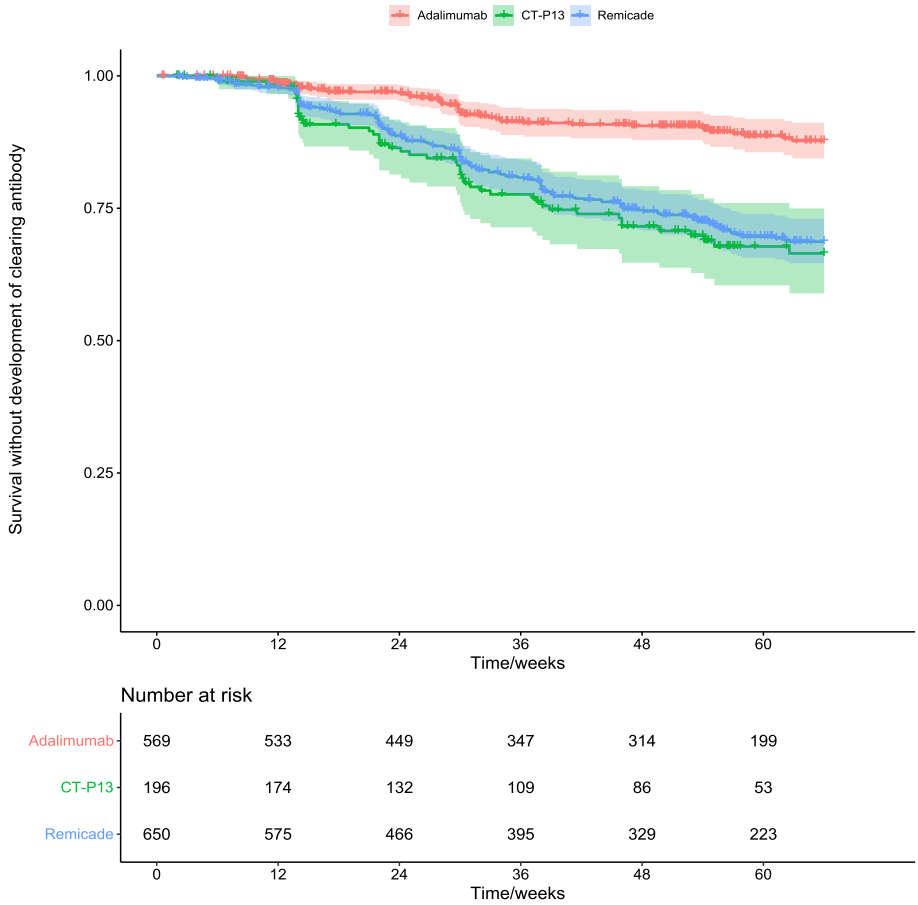

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Figure S18: Evolution of immunogenicity methotrexate vs thiopurines - antibody≥10AU/ml,

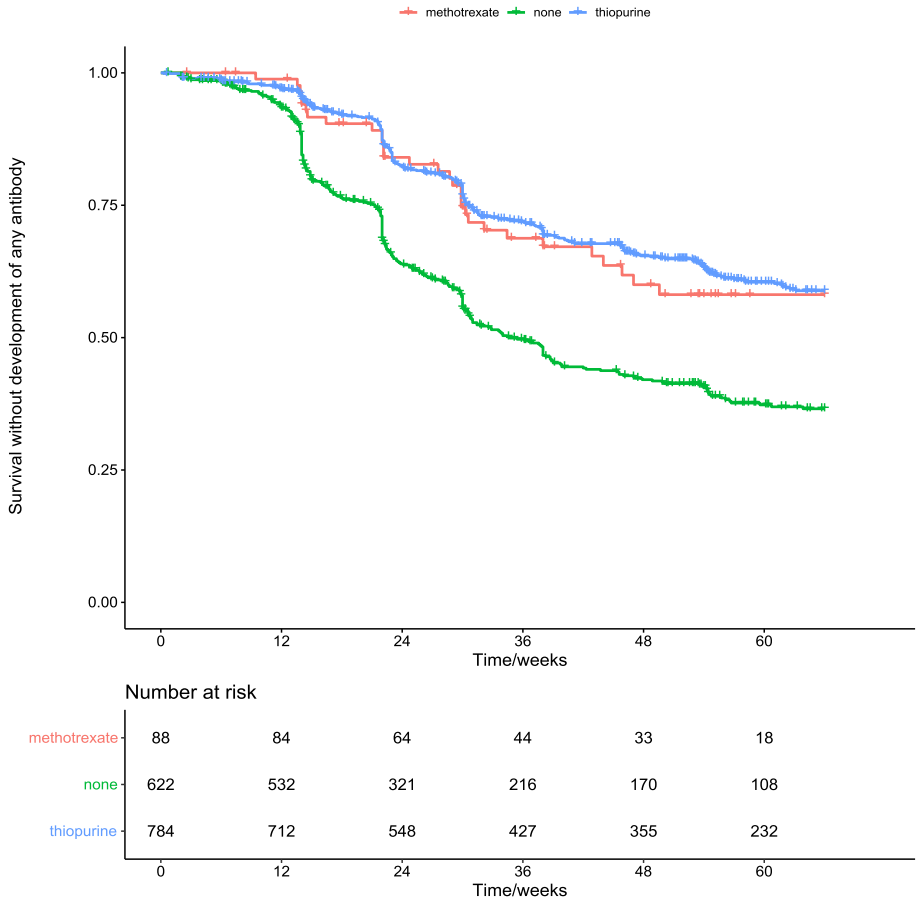

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Figure S19: Evolution of immunogenicity methotrexate vs thiopurines - antibody≥10AU/ml and undetectable drug)

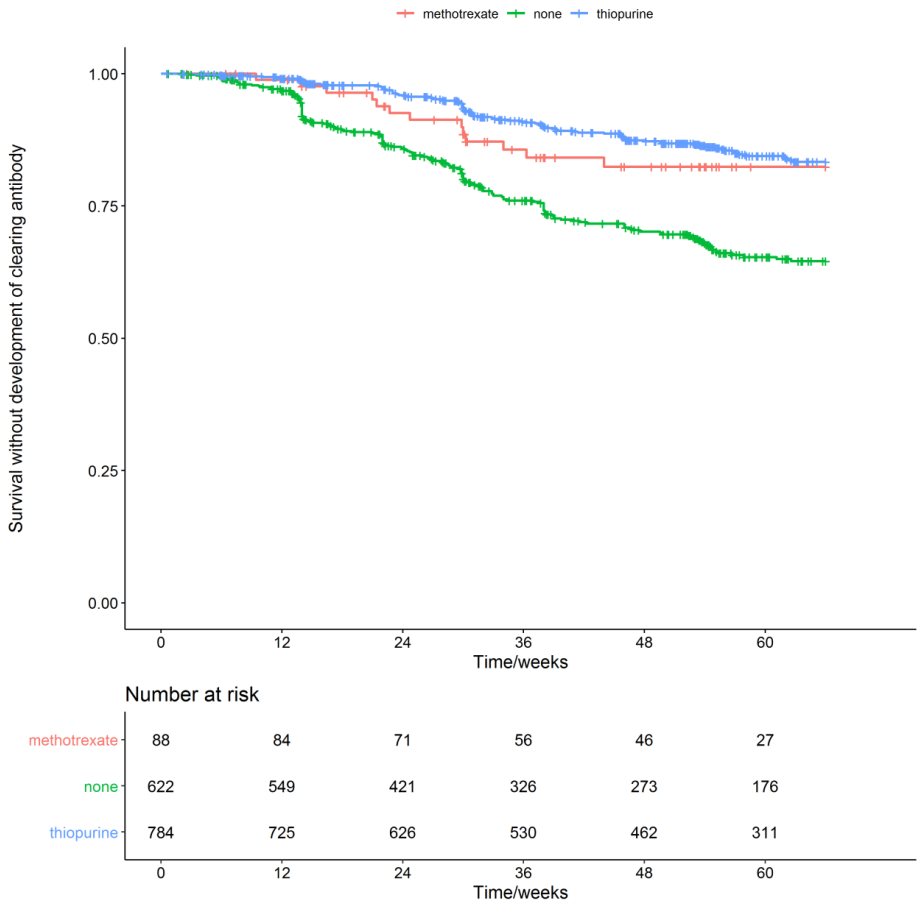

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Figure S20: Thiopurine dose and time to immunogenicity

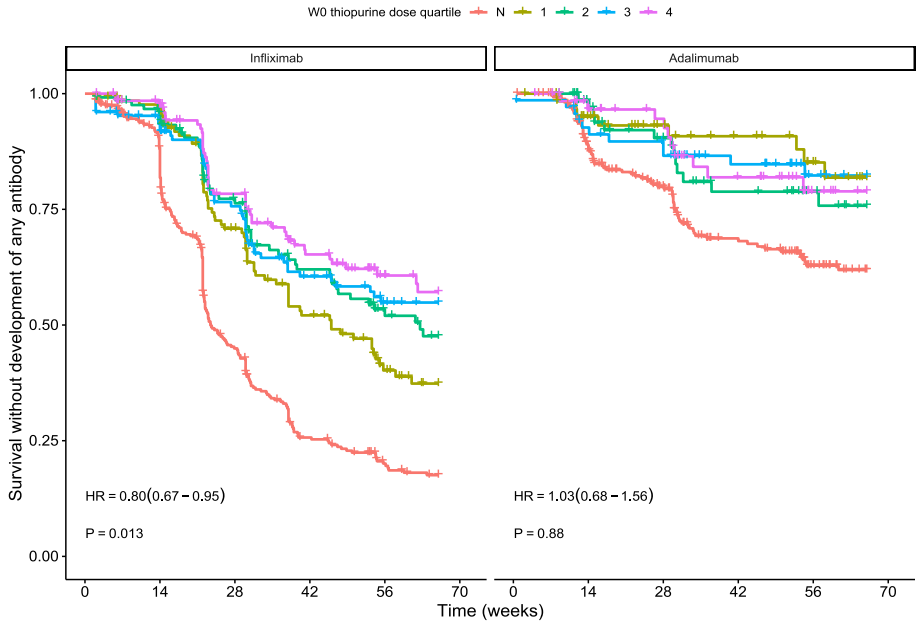
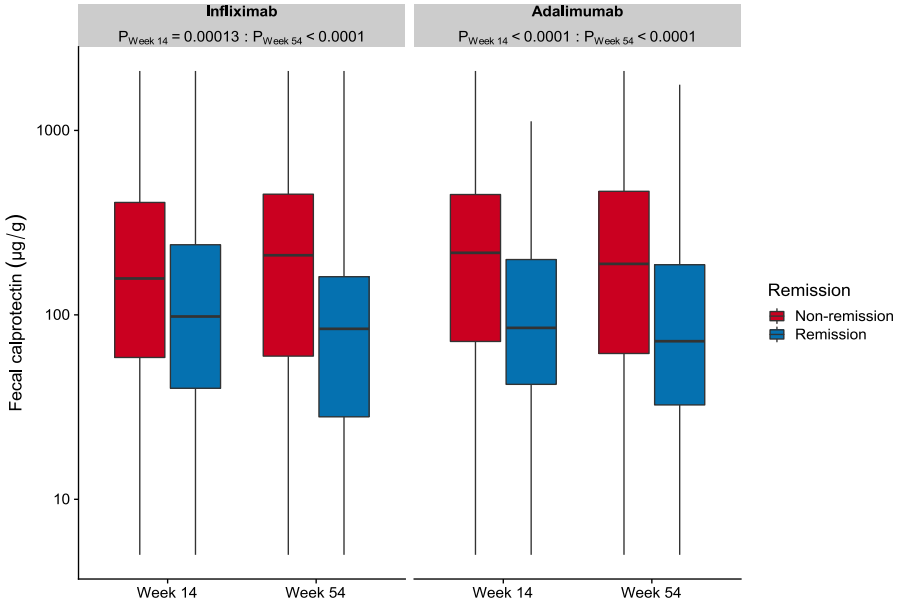


Figure S21: Week 14 and week 54 outcomes comparing CT-P13 and Remicade

Non-inferiority for biosimilar infliximab was assessed by determining whether the one-sided 95% confidence interval of the absolute difference in proportions was ≥10%. The confidence interval was calculated using the prop.test function in R.

Figure S22: Correlation of clinical outcomes and faecal calprotectin levels at weeks 14 and 54



Supplementary tables

Table S1 Baseline demographics, children excluded

Variable	Level	Infliximab n = 753	Adalimumab n = 638	p
Sex (male)		340 (45·2%)	310 (48·6%)	0·21
Age (years)		35·1 (26·4 - 48·0)	37·5 (28·6 - 50·0)	0·012
Ethnicity	**White**	696 (92·4%)	607 (95·1%)	0·085
	South Asian	26 (3·5%)	17 (2·7%)	
	Other	31 (4·1%)	14 (2·2%)	
Disease duration (years)		3·3 (0·8 - 11·4)	3·4 (0·8 - 11·5)	0·71
Age at diagnosis (years)		27·0 (20·8 - 38·2)	29·3 (21·8 - 41·1)	0·015
Montreal location	**L1**	224 (29·9%)	211 (33·7%)	0·092
	L2	201 (26·9%)	133 (21·2%)	
	L3	317 (42·4%)	277 (44·2%)	
	L4	6 (0·8%)	6 (1·0%)	
Montreal L4		32 (4·3%)	28 (4·5%)	0·90
Montreal behaviour	**B1**	421 (56·4%)	359 (57·2%)	0·00073
	B2	232 (31·1%)	227 (36·1%)	
	B3	93 (12·5%)	42 (6·7%)	
Perianal		116 (15·4%)	49 (7·7%)	<0·0001
Immunomodulator	**Azathioprine**	305 (40·6%)	253 (39·7%)	0·46
	Mercaptopurine	68 (9·0%)	46 (7·2%)	
	Methotrexate	40 (5·3%)	30 (4·7%)	
	None	339 (45·1%)	309 (48·4%)	
Steroids		210 (27·9%)	166 (26·0%)	0·47
Previous resectional surgery		201 (26·7%)	162 (25·4%)	0·62
HBI		6 (3 - 9)	5 (3 - 8)	0·0066
sPCDAI		10 (10 - 10)		
BMI		24·0 (21·1 - 28·3)	24·5 (21·5 - 28·3)	0·12
Hemoglobin (g/L)		128 (117 - 138)	131 (121 - 142)	<0·0001
White cell count ($\times 10^9$/L)		7·9 (6·1 - 10·1)	7·8 (6·2 - 9·9)	0·35
Platelet count ($\times 10^9$/L)		330 (274 - 401)	308 (256 - 384)	0·00081
Albumin (g/L)		39 (35 - 42)	39 (35 - 43)	0·073
CRP (mg/L)		8 (3 - 21)	6 (2 - 14)	0·00022
Fecal calprotectin (µg/g)		368 (134 - 751)	301 (127 - 632)	0·024

Median and interquartile range (IQR) are presented unless otherwise stated. The significance of differences between continuous variables was calculated using the Mann-Whitney U test. Differences between categorical variables were sought using Fisher's exact test was used.

Table S2 Baseline demographics effectiveness cohort only

Variable	Level	infliximab n = 898	adalimumab n = 605	p
Sex (male)		439 (48·9%)	297 (49·1%)	0·96
Age (years)		29·8 (19·2 - 44·0)	37·0 (27·4 - 49·5)	<0·0001
Ethnicity	White	808 (90·0%)	577 (95·4%)	0·00050
	South Asian	46 (5·1%)	15 (2·5%)	
	Other	44 (4·9%)	13 (2·1%)	
Disease duration (years)		2·2 (0·6 - 7·5)	3·0 (0·7 - 10·6)	0·001
Age at diagnosis (years)		23·9 (15·7 - 34·2)	29·0 (21·5 - 41·0)	<0·0001
Montreal location	L1	238 (26·7%)	201 (33·7%)	0·027
	L2	224 (25·1%)	127 (21·3%)	
	L3	421 (47·3%)	262 (44·0%)	
	L4	8 (0·9%)	6 (1·0%)	
Montreal L4		112 (12·6%)	31 (5·2%)	<0·0001
Montreal behaviour	B1	566 (63·5%)	351 (58·9%)	<0·0001
	B2	232 (26·0%)	210 (35·2%)	
	B3	93 (10·4%)	35 (5·9%)	
Perianal		134 (14·9%)	48 (7·9%)	<0·0001
Immunomodulator	azathioprine	420 (46·9%)	248 (41·0%)	0·010
	mercaptopurine	74 (8·3%)	44 (7·3%)	
	methotrexate	55 (6·1%)	27 (4·5%)	
	none	347 (38·7%)	286 (47·3%)	
Steroids		268 (29·8%)	158 (26·1%)	0·13
Previous resectional surgery		169 (18·8%)	131 (21·7%)	0·19
HBI		6·0 (3·0 - 9·0)	5·0 (3·0 - 8·0)	0·060
sPCDAI		30·0 (15·0 - 50·0)		
BMI		22·7 (19·5 - 26·9)	24·4 (21·5 - 28·2)	<0·0001
Hemoglobin (g/L)		125·0 (114·0 - 135·0)	131·0 (120·0 - 142·0)	<0·0001
White cell count ($\times 10^9$/L)		8·0 (6·2 - 10·3)	7·8 (6·2 - 9·8)	0·052
Platelet count ($\times 10^9$/L)		344·5 (284·0 - 417·0)	311·0 (258·0 - 386·0)	<0·0001
Albumin (g/L)		38·0 (34·0 - 42·0)	39·0 (35·0 - 43·0)	0·003
CRP (mg/L)		9·0 (3·0 - 25·0)	7·0 (2·0 - 14·0)	<0·0001
Fecal calprotectin (µg/g)		437 (187 - 892)	320 (141 - 661)	<0·0001

Median and interquartile range (IQR) are presented unless otherwise stated. The significance of differences between continuous variables was calculated using the Mann-Whitney U test. Differences between categorical variables were sought using Fisher's exact test.

Table S3 Univariable associations with primary non-response at week 14

Variable	Level	Infliximab			Adalimumab		
Week 14 outcome		**PNR n = 170**	**Not PNR n = 605**	**p**	**PNR n = 125**	**Not PNR n = 341**	**p**
Sex (male)		72 (42·4%)	306 (50·6%)	0·068	63 (50·4%)	167 (49·0%)	0·83
Age (years)		33·7 (22·5 - 49·1)	29·0 (18·4 - 42·5)	0·0048	38·6 (26·6 - 55·0)	36·3 (28·6 - 49·2)	0·30
Disease duration (years)		1·5 (0·5 - 6·5)	2·3 (0·7 - 7·7)	0·11	1·6 (0·6 - 10·6)	3·3 (0·8 - 10·9)	0·15
Baseline immunomodulator		85 (50·0%)	392 (64·8%)	0·00067	58 (46·4%)	193 (56·6%)	0·059
Montreal location	L1	47 (27·6%)	159 (26·5%)	0·98	40 (32·0%)	106 (31·7%)	0·92
	L2	40 (23·5%)	146 (24·3%)		25 (20·0%)	75 (22·5%)	
	L3	82 (48·2%)	289 (48·2%)		59 (47·2%)	151 (45·2%)	
	L4	1 (0·6%)	6 (1·0%)		1 (0·8%)	2 (0·6%)	
Montreal behaviour	B1	110 (65·9%)	387 (64·2%)	0·92	75 (60·5%)	186 (55·2%)	0·63
	B2	41 (24·6%)	151 (25·0%)		42 (33·9%)	130 (38·6%)	
	B3	16 (9·6%)	65 (10·8%)		7 (5·6%)	21 (6·2%)	
Perianal disease ever		22 (12·9%)	100 (16·5%)	0·28	10 (8·0%)	26 (7·6%)	0·85
Previous resectional surgery		32 (18·8%)	109 (18·0%)	0·82	28 (22·4%)	74 (21·7%)	0·90
Charlson comorbidity score	0	149 (87·6%)	559 (92·4%)	0·14	106 (84·8%)	312 (91·5%)	0·088
	1	16 (9·4%)	35 (5·8%)		13 (10·4%)	21 (6·2%)	
	≥2	5 (2·9%)	11 (1·8%)		6 (4·8%)	8 (2·3%)	
Baseline BMI category	Normal	73 (42·9%)	291 (48·1%)	0·29	62 (49·6%)	164 (48·1%)	0·0027
	Underweight	31 (18·2%)	105 (17·4%)		5 (4·0%)	25 (7·3%)	
	Overweight	35 (20·6%)	132 (21·8%)		25 (20·0%)	106 (31·1%)	
	Obese	31 (18·2%)	77 (12·7%)		33 (26·4%)	46 (13·5%)	
Baseline current smoker		41 (24·4%)	84 (14·0%)	0·0020	25 (20·2%)	62 (18·4%)	0·69
Baseline haemoglobin (g/L)		122 (111 - 135)	125 (115 - 135)	0·088	130 (120 - 139)	131 (120 - 142)	0·48
Baseline white cell count ($\times 10^9$/L)		8·9 (7·0 - 10·9)	7·9 (6·1 - 10·0)	0·0011	8·2 (6·5 - 10·4)	7·7 (6·1 - 9·6)	0·052
Baseline platelet count ($\times 10^9$/L)		363 (290 - 436)	343 (284 - 412)	0·10	311 (267 - 393)	307 (251 - 384)	0·36
Baseline albumin (g/L)		37 (32 - 41)	39 (34 - 42)	0·0092	39 (34 - 43)	39 (36 - 43)	0·48
Baseline faecal calprotectin (µg/g)		501 (194 - 960)	430 (192 - 898)	0·61	277 (139 - 600)	338 (158 - 688)	0·21
Week 14 drug level (mg/L)		2·3 (0·9 - 5·0)	4·0 (1·9 - 7·2)	0·00013	8·4 (4·4 - 11·3)	11·6 (8·4 - 15·3)	<0·0001
Week 14 antibody level (AU/mL)		5·0 (3·0 - 9·0)	4·0 (2·0 - 6·0)	0·00039	3·0 (2·0 - 6·0)	2·0 (2·0 - 3·1)	0·0010

Median and interquartile range (IQR) or numbers (%) are presented. The significance of differences between continuous variables was calculated using the Mann-Whitney U test. Differences between categorical variables were sought using Fisher's exact test. PNR: primary non response.

Table S4a Predictive model of PNR for infliximab patients

Term	Odds ratio (95% confidence interval)	p value	Frequency
Intercept	1·31 (0·22-7·5)	0·76	1
Baseline Immunomodulators	0·67 (0·42-1·07)	0·093	0·94
Current Smoker	1·71 (0·98-2·94)	0·056	1
Baseline Haemoglobin	0·98 (0·97-1)	0·033	0·98
Age at First Dose	1·02 (1-1·03)	0·036	0·98
Disease Duration at First Dose	0·97 (0·94-1·01)	0·12	0·98
Prior Surgery	NA	NA	0
Baseline BMI	NA	NA	0
Baseline Albumin	NA	NA	0·02
Baseline White Cell Count	NA	NA	0·07
Baseline Calprotectin	NA	NA	0

Table S4b Predictive model of PNR for adalimumab patients

Term	Odds ratio (95% confidence interval)	p value	Frequency
Intercept	0·21 (0·02-2·44)	0·22	1
Baseline BMI	1·07 (1·02-1·12)	0·0046	1
Baseline White Cell Count	1·08 (0·99-1·18)	0·076	1
Baseline Haemoglobin	0·99 (0·97-1)	0·11	0·99
Baseline Immunomodulators	NA	NA	0
Current Smoker	NA	NA	0
Age at First Dose	NA	NA	0
Disease Duration at First Dose	NA	NA	0
Prior Surgery	NA	NA	0
Baseline Albumin	NA	NA	0

The above tables show the coefficients for the predictive models of PNR using baseline data. Estimate and p-value are calculated from using the full dataset to fit the model, and shaded variables survived to the end under model selection using AIC. The frequency column shows the frequency with which each variable survived model selection during the leave-one-out cross-validation process.

Table S5 Univariable associations with non-remission at week 54 (excluding patients who exited for primary non-response)

		Infliximab			Adalimumab		
Week 54 outcome		**Non-remission n = 364**	**Remission n = 279**	**p**	**Non-remission n = 214**	**Remission n = 130**	**p**
Sex (male)		166 (45·6%)	161 (57·7%)	0·0025	115 (53·7%)	62 (47·7%)	0·32
Age (years)		32·0 (21·1 - 46·2)	27·0 (17·6 - 38·9)	0·00043	38·9 (29·2 - 51·5)	36·1 (27·6 - 51·0)	0·32
Disease duration (years)		2·5 (0·6 - 7·8)	2·1 (0·6 - 6·8)	0·37	3·8 (0·9 - 12·2)	3·0 (0·8 - 9·2)	0·18
Baseline immunomodulator		196 (53·8%)	209 (74·9%)	<0·0001	112 (52·3%)	75 (57·7%)	0·37
Montreal location	L1	101 (28·0%)	71 (25·6%)	0·70	65 (30·8%)	39 (30·7%)	0·16
	L2	83 (23·0%)	71 (25·6%)		42 (19·9%)	37 (29·1%)	
	L3	174 (48·2%)	131 (47·3%)		102 (48·3%)	51 (40·2%)	
	L4	3 (0·8%)	4 (1·4%)		2 (0·9%)	0 (0·0%)	
Montreal behaviour	B1	228 (63·0%)	185 (66·5%)	0·65	121 (56·5%)	74 (58·3%)	0·41
	B2	94 (26·0%)	65 (23·4%)		84 (39·3%)	44 (34·6%)	
	B3	40 (11·0%)	28 (10·1%)		9 (4·2%)	9 (7·1%)	
Perianal disease ever		50 (13·7%)	56 (20·1%)	0·041	9 (4·2%)	14 (10·8%)	0·025
Previous resectional surgery		74 (20·3%)	42 (15·1%)	0·098	45 (21·0%)	30 (23·1%)	0·69
Charlson comorbidity score	0	327 (89·8%)	261 (93·5%)	0·14	189 (88·3%)	118 (90·8%)	0·80
	1	28 (7·7%)	16 (5·7%)		19 (8·9%)	9 (6·9%)	
	≥2	9 (2·5%)	2 (0·7%)		6 (2·8%)	3 (2·3%)	
Baseline BMI category	Normal	153 (42·0%)	144 (51·6%)	0·00022	89 (41·6%)	81 (62·3%)	<0·0001
	Underweight	58 (15·9%)	60 (21·5%)		10 (4·7%)	10 (7·7%)	
	Overweight	89 (24·5%)	53 (19·0%)		67 (31·3%)	29 (22·3%)	
	Obese	64 (17·6%)	22 (7·9%)		48 (22·4%)	10 (7·7%)	
Baseline current smoker		64 (17·8%)	30 (10·8%)	0·013	47 (22·2%)	15 (11·6%)	0·014
Baseline haemoglobin (g/L)		124 (113 - 135)	126 (115 - 135)	0·20	133 (120 - 143)	131 (120 - 140)	0·45
Baseline white cell count ($\times 10^9$/L)		8·6 (6·6 - 10·8)	7·4 (5·7 - 9·5)	<0·0001	8·3 (6·3 - 9·8)	7·5 (5·7 - 10·2)	0·15
Baseline platelet count ($\times 10^9$/L)		348 (286 - 417)	331 (280 - 402)	0·23	313 (258 - 394)	299 (245 - 384)	0·39
Baseline albumin (g/L)		38 (34 - 42)	39 (34 - 43)	0·083	39 (35 - 42)	40 (35 - 43)	0·46
Baseline faecal calprotectin (µg/g)		432 (165 - 858)	446 (222 - 944)	0·54	366 (180 - 801)	313 (141 - 591)	0·095
Week 14 drug level (mg/L)		2·9 (1·2 - 5·7)	5·3 (2·8 - 8·8)	<0·0001	9·2 (7·0 - 12·5)	13·3 (10·7 - 17·8)	<0·0001
Week 14 antibody level (AU/mL)		4·0 (3·0 - 9·0)	3·0 (2·0 - 5·0)	<0·0001	3·0 (2·0 - 4·0)	2·0 (2·0 - 3·0)	0·011
Week 14 status	Remission	104 (29·5%)	188 (70·7%)	<0·0001	63 (31·2%)	94 (74·0%)	<0·0001
	Response	78 (22·1%)	28 (10·5%)		37 (18·3%)	12 (9·4%)	
	Grey zone	100 (28·3%)	40 (15·0%)		50 (24·8%)	17 (13·4%)	
	PNR	71 (20·1%)	10 (3·8%)		52 (25·7%)	4 (3·1%)	
Immunogenicity in first year	Antibody -ve	137 (37·6%)	142 (50·9%)	<0·0001	155 (73·8%)	112 (86·2%)	0·012
	Antibody +ve drug +ve	94 (25·8%)	97 (34·8%)		37 (17·6%)	15 (11·5%)	
	Antibody +ve drug -ve	133 (36·5%)	40 (14·3%)		18 (8·6%)	3 (2·3%)	

Median and interquartile range (IQR) or numbers (%) are presented. The significance of differences between continuous variables was calculated using the Mann-Whitney U test. Differences between categorical variables were sought using Fisher's exact test. PNR: primary non response.

Table S6a Predictive model of week 54 remission prior to treatment for infliximab patients

Term	Odds ratio (95% confidence interval)	p value	Frequency
Intercept	3·27 (0·49-22·59)	0·22	1
Baseline Immunomodulators	0·51 (0·33-0·78)	0·0021	1
Baseline CRP	1·42 (1·01-2)	0·043	1
Baseline BMI	1·06 (1·02-1·10)	0·0018	1
Baseline Haemoglobin	0·98 (0·97-1·00)	0·022	1
Current Smoker	1·67 (0·97-2·95)	0·070	1
Baseline White Cell Count	NA	NA	0·02
Age at First Dose	NA	NA	0
Disease Duration at First Dose	NA	NA	0
Prior Surgery	NA	NA	0
Baseline Albumin	NA	NA	0

Table S6b Predictive model of week 54 remission prior to treatment for adalimumab patients

Term	Odds ratio (95% confidence interval)	p value	Frequency
Intercept	0·03 (0-0·12)	<0·0001	1
Baseline BMI	1·14 (1·07-1·21)	<0·0001	1
Baseline CRP	2·67 (1·67-4·35)	<0·0001	1
Current Smoker	2·23 (1·04-5·07)	0·045	1
Disease Duration at First Dose	1·03 (1-1·06)	0·099	1
Baseline Immunomodulators	NA	NA	0
Baseline Haemoglobin	NA	NA	0
Baseline White Cell Count	NA	NA	0
Age at First Dose	NA	NA	0
Prior Surgery	NA	NA	0
Baseline Albumin	NA	NA	0

The above tables show the coefficients for the predictive models of week 54 remission using baseline data. Estimate and p-value are calculated from using the full dataset to fit the model, and shaded variables survived to the end under model selection using AIC. The frequency column shows the frequency with which each variable survived model selection during the leave-one-out cross-validation process.

Table S7a Predictive model of week 54 non-remission after 14 weeks for infliximab patients

Term	Odds ratio (95% confidence interval)	p value	Frequency
Intercept	0·52 (0·01-20·6)	0·73	1
Week 14 Remission Status	0·16 (0·08-0·31)	<0·0001	1
Week 14 White Cell Count	1·27 (1·06-1·54)	0·012	1
Age at First Dose	1·03 (1·01-1·06)	0·0067	1
Week 14 Haemoglobin	0·97 (0·95-0·99)	0·017	1
Prior Surgery	0·35 (0·15-0·82)	0·017	1
Week 14 Anti-drug Antibody Level	2·34 (1·11-5·4)	0·033	1
Week 14 Calpro	1·70 (0·97-3·05)	0·066	1
Baseline BMI	1·07 (1-1·15)	0·071	1
Disease Duration at First Dose	NA	NA	0
Baseline CRP	NA	NA	0
Baseline Immunomodulators	NA	NA	0
Current Smoker	NA	NA	0
Baseline Haemoglobin	NA	NA	0
Baseline Albumin	NA	NA	0
Baseline White Cell Count	NA	NA	0
Baseline Calprotectin	NA	NA	0
Week 14 Drug Level	NA	NA	0
Week 14 Albumin	NA	NA	0
Week 14 CRP	NA	NA	0

The model is given by the odds ratios in a logistic regression formula:

$$\ln\left(\frac{p}{1-p}\right) = -0\cdot 645 - 1\cdot 844x_1 + 0\cdot 237x_2 + 0\cdot 032x_3 - 0\cdot 027x_4 - 1\cdot 042x_5 + 0\cdot 852x_6 + 0\cdot 533x_7 + 0\cdot 065x_8$$

Where x_1 is week 14 remission status (1 for remission, 0 for non-remission), x_2 is week 14 white cell count, x_3 is age at first dose, x_4 is week 14 haemoglobin, x_5 is prior surgeries (1 for yes, 0 for no), x_6 is log10 week 14 antibody level, x_7 is log10 week 14 calprotectin and x_8 is baseline BMI.

For example, for a patient not in remission, with white cell count 5·80, age 30, haemoglobin 148, no prior surgery, antibody level 20, calprotectin 100, baseline BMI 25:

$$\ln\left(\frac{p}{1-p}\right) = -0\cdot 645 + 0\cdot 237 * 5\cdot 8 + 0\cdot 032 * 30 - 0\cdot 027 * 148 + 0\cdot 852 * \log_{10}(20) + 0\cdot 533 * \log_{10}(100) + 0\cdot 065 * 25$$

$$\ln\left(\frac{p}{1-p}\right) = 1\cdot 49$$

then

$$p = \frac{e^{1\cdot 49}}{1+e^{1\cdot 49}} = \mathbf{0.81}$$

So, the patient has an 81% chance of treatment failure. If the patient was in remission at week 14, the estimated chance would have been only 41%. As per supplementary figure S5c, 55·9% is the threshold at which we determine a binary prediction from the probabilistic model outcome.

Table S7b Predictive model of week 54 non-remission after 14 weeks for adalimumab patients

Term	Odds ratio (95% confidence interval)	p value	Frequency
Intercept	0·3 (0·01-15·56)	0·55	1
Week 14 CRP	3·82 (1·51-10·22)	0·0058	1
Week 14 Drug Level	0·06 (0·01-0·38)	0·0056	1
Week 14 Haemoglobin	1·03 (1·01-1·06)	0·013	1
Week 14 Remission Status	0·43 (0·18-1·04)	0·061	1
Current Smoker	2·69 (0·96-8·15)	0·068	0·99
Disease Duration at First Dose	1·04 (1-1·08)	0·079	1
Week 14 White Cell Count	NA	NA	0·03
Age at First Dose	NA	NA	0
Prior Surgery	NA	NA	0
Week 14 Calprotectin	NA	NA	0
Baseline BMI	NA	NA	0·01
Baseline CRP	NA	NA	0·02
Baseline Immunomodulators	NA	NA	0
Baseline Haemoglobin	NA	NA	0·02
Baseline Albumin	NA	NA	0
Baseline White Cell Count	NA	NA	0·01
Baseline Calprotectin	NA	NA	0
Week 14 Albumin	NA	NA	0
Week 14 CRP	NA	NA	0
Week 14 Anti-drug Antibody Level	NA	NA	0·01

The model for adalimumab patients is given by

$$\ln\left(\frac{p}{1-p}\right) = -1 \cdot 889 + 1 \cdot 339x_1 - 2 \cdot 865x_2 + 0 \cdot 031x_3 - 0 \cdot 84x_4 + 0 \cdot 99x_5 + 0 \cdot 034$$

Where x_1 is log10 week 14 CRP, x_2 is log10 week 14 drug level, x_3 week 14 haemoglobin, x_4 is week 14 remission status, x_5 is current smoker status and x_6 is disease duration at first dose

Table S8a Adverse events

	Infliximab			Adalimumab n=655
	Remicade (n=753)	**CT-P13** (n=202)	**Any infliximab** (n=955)	
Any adverse event	106 (52·5%)	395 (52·5%)	501 (52·5%)	254 (38·8%)
Any adverse event (excluding worsening of CD activity)	102 (50·5%)	370 (49·1%)	472 (49·4%)	233 (35·6%)
Any serious adverse event	38 (18·8%)	159 (21·1%)	197 (20·6%)	115 (17·6%)
Any serious infection	12 (5·9%)	26 (3·5%)	38 (4·0%)	21 (3·2%)
Any serious adverse event (excluding worsening of CD activity)	35 (17·3%)	136 (18·1%)	171 (17·9%)	96 (14·7%)
Any event requiring hospital admission	31 (15·3%)	138 (18·3%)	169 (17·7%)	105 (16·0%)
Any event requiring hospital admission (excluding worsening of CD as the sole reason)	27 (13·4%)	113 (15·0%)	140 (14·7%)	86 (13·1%)
Any adverse event leading to drug withdrawal (excluding worsening of CD activity)	16 (7·9%)	68 (9·0%)	84 (8·8%)	42 (6·4%)
Death	1 (0·5%)	2 (0·3%)	3 (0·3%)	2 (0·3%)

Table S8b Adverse Events of Special Interest

	Infliximab			Adalimumab n=655
	Remicade (n=753)	**CT-P13** (n=202)	**Any infliximab** (n=955)	
All infections	115 (15·3%)	30 (14·9%)	145 (15·2%)	58 (8·9%)
Lower respiratory tract infections	28 (3·7%)	6 (3·0%)	34 (3·6%)	10 (1·5%)
Upper respiratory tract infections	14 (1·9%)	2 (1·0%)	16 (1·7%)	11 (1·7%)
Urinary tract infections	11 (1·5%)	3 (1·5%)	14 (1·5%)	3 (0·5%)
Infectious gastroenteritis	6 (0·8%)	2 (1·0%)	8 (0·8%)	4 (0·6%)
Tuberculosis	1 (0·1%)	2 (1·0%)	3 (0·3%)	
Adverse skin reaction	68 (9·0%)	18 (8·9%)	86 (9·0%)	33 (5·0%)
Infusion/Injection reaction	26 (3·5%)	5 (2·5%)	31 (3·2%)	
Injection site reaction				28 (4·3%)
Headache	33 (4·4%)	9 (4·5%)	42 (4·4%)	24 (3·7%)
Nausea/vomiting	29 (3·9%)	9 (4·5%)	38 (4·0%)	9 (1·4%)
Fatigue/lethargy/malaise	21 (2·8%)	8 (4·0%)	29 (3·0%)	4 (0·6%)
Paraesthesias and dysaesthesias	9 (1·2%)	2 (1·0%)	11 (1·2%)	6 (0·9%)
Lupus-like syndrome	4 (0·5%)		4 (0·4%)	2 (0·3%)
Cancer	2 (0·3%)		2 (0·2%)	1 (0·2%)

Table S9 Risk of infections stratified by concomitant immunomodulator use and age

Variable	Odds ratio (95% CI)	p
Age >= 50 years	1·42 (0·74 - 2·56)	0·27
Week 0 immunomodulator	0·89 (0·52 - 1·52)	0·65
Adalimumab (vs. infliximab)	0·77 (0·44 - 1·31)	0·34

Odds ratios and *P* values calculated using logistic regression.

Table S10 Univariable associations with drug concentration at week 14

Variable		Infliximab	p	Adalimumab	p
Age (years)		rho = -0·05	0·25	rho = -0·13	0·010
Disease duration (years)		rho = 0·10	0·014	rho = -0·00	0·94
Baseline immunomodulator	FALSE	2·70 (1·12 - 5·88)	<0·0001	10·20 (7·00 - 13·40)	0·039
	TRUE	4·40 (2·30 - 7·50)		11·20 (7·90 - 14·55)	
Baseline BMI category	Normal	3·90 (1·80 - 7·03)	0·0014	12·00 (8·70 - 15·90)	<0·0001
	Underweight	2·60 (1·20 - 4·83)		12·35 (8·25 - 17·77)	
	Overweight	4·90 (2·00 - 8·00)		10·40 (7·25 - 12·75)	
	Obese	3·30 (1·65 - 6·70)		8·40 (5·00 - 11·25)	
Baseline current smoker	FALSE	3·90 (1·80 - 7·20)	0·015	11·00 (7·70 - 14·45)	0·054
	TRUE	3·10 (1·20 - 5·70)		10·00 (6·30 - 12·90)	
Baseline haemoglobin (g/L)		rho = 0·13	0·0029	rho = -0·01	0·85
Baseline white cell count (×10^9/L)		rho = -0·12	0·0064	rho = -0·06	0·28
Baseline platelet count (×10^9/L)		rho = -0·17	<0·0001	rho = -0·02	0·77
Baseline albumin (g/L)		rho = 0·20	<0·0001	rho = 0·05	0·34
Baseline faecal calprotectin (µg/g)		rho = -0·15	0·0032	rho = -0·14	0·014
Week 14 antibody level (AU/mL)		rho = -0·35	<0·0001	rho = -0·40	<0·0001
Baseline HBI/sPCDAI non-remission	FALSE	4·40 (2·05 - 7·85)	0·041	11·90 (8·50 - 15·90)	0·0013
	TRUE	3·60 (1·65 - 6·55)		10·20 (7·00 - 13·50)	
Baseline CRP (mg/L)		rho = -0·19	<0·0001	rho = -0·20	<0·0001
Week 14 HBI/sPCDAI non-remission	FALSE	4·10 (1·80 - 7·47)	0·0027	11·50 (8·53 - 15·30)	<0·0001
	TRUE	3·00 (1·20 - 5·80)		8·40 (5·53 - 11·60)	
Week 14 CRP (mg/L)		rho = -0·36	<0·0001	rho = -0·37	<0·0001
Week 14 faecal calprotectin (µg/g)		rho = -0·40	<0·0001	rho = -0·35	<0·0001
Week 14 albumin (g/L)		rho = 0·30	<0·0001	rho = 0·18	0·00076
Week 14 status	Remission	5·25 (2·90 - 8·60)	<0·0001	13·00 (9·52 - 16·90)	<0·0001
	Response	2·55 (1·10 - 4·82)		10·40 (7·00 - 12·17)	
	Grey zone	2·70 (1·20 - 5·70)		10·10 (7·45 - 12·93)	
	PNR	2·35 (0·92 - 4·95)		8·40 (4·40 - 11·27)	

For categorical variables, the median and interquartile range for each level are shown and the p value is calculated using Mann Whitney U tests. For continuous variables, the Spearman's rho correlation coefficient is shown with its associated p value. BMI: body mass index; CRP: C-reactive protein; HBI: Harvey Bradshaw index; sPCDAI: short paediatric Crohn's disease index.

Table S11 Univariable associations with drug concentration at week 54

Variable	Level	Infliximab	p	Adalimumab	p
Baseline immunomodulator	FALSE	1·60 (0·70 - 4·07)	<0·0001	9·70 (5·10 - 13·45)	0·21
	TRUE	3·20 (1·50 - 5·47)		10·30 (6·30 - 14·30)	
Previous resectional surgery	FALSE	2·50 (1·12 - 5·00)	0·92	10·30 (6·23 - 14·30)	0·014
	TRUE	3·10 (0·70 - 5·55)		8·35 (3·12 - 12·38)	
Baseline BMI category	Normal	3·05 (1·48 - 5·60)	0·036	11·70 (6·80 - 15·30)	0·00066
	Underweight	2·40 (1·20 - 4·73)		10·70 (4·25 - 13·35)	
	Overweight	2·30 (0·70 - 4·85)		9·30 (5·90 - 12·45)	
	Obese	2·20 (0·70 - 4·10)		7·00 (4·40 - 9·80)	
Baseline current smoker	FALSE	2·70 (1·20 - 5·20)	0·070	10·45 (6·23 - 14·40)	0·00072
	TRUE	2·30 (0·70 - 4·32)		7·70 (4·38 - 11·53)	
Baseline platelet count ($\times 10^9$/L)		rho = -0·11	0·018	rho = -0·03	0·60
Week 14 drug level (mg/L)		rho = 0·51	<0·0001	rho = 0·41	<0·0001
Week 14 antibody level (AU/mL)		rho = -0·17	0·00053	rho = -0·30	<0·0001
Week 14 HBI/sPCDAI non-remission	FALSE	2·70 (1·10 - 5·20)	0·12	10·60 (6·38 - 14·40)	0·020
	TRUE	2·30 (0·72 - 4·60)		8·70 (4·30 - 12·55)	
Week 14 CRP (mg/L)		rho = -0·20	<0·0001	rho = -0·20	0·00081
Week 14 faecal calprotectin (µg/g)		rho = -0·27	<0·0001	rho = 0·00	0·98
Week 14 albumin (g/L)		rho = 0·15	0·0021	rho = 0·17	0·0087
Week 14 status	Remission	3·00 (1·40 - 5·40)	0·0092	11·15 (7·50 - 14·93)	0·018
	Response	1·85 (0·70 - 3·40)		11·00 (6·90 - 14·70)	
	Grey zone	2·50 (1·02 - 5·60)		10·40 (5·95 - 14·05)	
	PNR	2·00 (0·85 - 4·30)		7·30 (3·60 - 10·80)	
Week 54 HBI/sPCDAI non-remission	FALSE	2·90 (1·20 - 5·35)	0·0016	10·40 (5·90 - 14·30)	0·020
	TRUE	1·60 (0·70 - 4·00)		7·90 (4·40 - 11·80)	
Week 54 CRP (mg/L)		rho = -0·37	<0·0001	rho = -0·40	<0·0001
Week 54 faecal calprotectin (µg/g)		rho = -0·41	<0·0001	rho = -0·27	<0·0001
Week 54 albumin (g/L)		rho = 0·22	<0·0001	rho = 0·21	0·00044

For categorical variables, the median and interquartile range for each level are shown and the p value is calculated using Mann Whitney U tests. For continuous variables, the Spearman's rho correlation coefficient is shown with its associated p value. BMI: body mass index; CRP: C-reactive protein; HBI: Harvey Bradshaw index; sPCDAI: short paediatric Crohn's disease index.

Table S12 Univariable associations with development of immunogenicity (antibody ≥ 10 AU/mL)

Variable		Infliximab (n = 925)		Adalimumab (n = 569)	
		Hazard ratio (95% CI)	p	Hazard ratio (95% CI)	p
Age (years)		1·01 (1·00 - 1·01)	0·0058	1·01 (1·00 - 1·02)	0·12
Baseline immunomodulator		0·39 (0·32 - 0·46)	<0·0001	0·44 (0·31 - 0·64)	<0·0001
Baseline BMI category	Normal	Reference		Reference	
	Underweight	1·04 (0·80 - 1·34)	0·79	1·20 (0·57 - 2·54)	0·63
	Overweight	1·08 (0·86 - 1·37)	0·50	1·15 (0·75 - 1·78)	0·53
	Obese	1·55 (1·18 - 2·02)	0·0014	2·47 (1·60 - 3·82)	<0·0001
Baseline current smoker		1·69 (1·33 - 2·14)	<0·0001	1·46 (0·98 - 2·18)	0·064
Baseline white cell count ($\times 10^9$/L)		1·05 (1·02 - 1·08)	0·00031	1·05 (0·99 - 1·11)	0·11
Log_{10}(week 14 drug level (mg/L))[a]		0·43 (0·30 - 0·61)	<0·0001	0·10 (0·03 - 0·29)	<0·0001

Hazard ratios and p values calculated using Cox proportional hazards. BMI: body mass index; CI: confidence interval

[a] Hazard ratios are displayed for each ten-fold increase in week 14 drug level. For this specific analysis, patients who developed positive anti-drug antibodies prior to or at their week 14 drug level, or who had no further antibody concentrations following week 14, were excluded from analysis.

Table S13 Impact of thiopurine therapy, drug level and immunogenicity on non-remission at week 54

Variable	Odds ratio (95% CI)	p
Log_{10}(week 14 drug level (mg/L))	0·30 (0·18 - 0·49)	<0·0001
Log_{10}(week 14 antibody level (AU/mL))	1·61 (1·02 - 2·63)	0·048
Baseline immunomodulator	0·56 (0·38 - 0·83)	0·004

Odds ratios and *P* values calculated using logistic regression. Odds ratios for drug and antibody levels represent the change in odds for each ten-fold change in the respective variable.

Table S14 Baseline demographics Remicade vs CT-P13

Variable	Level	Remicade n = 674	CT-P13 n = 202	p
Sex (male)		326 (48·4%)	101 (50·0%)	0·69
Age (years)		29·5 (18·5 - 43·3)	31·3 (21·6 - 48·0)	0·024
Ethnicity	White	608 (90·2%)	180 (89·1%)	0·076
	South Asian	39 (5·8%)	7 (3·5%)	
	Other	27 (4·0%)	15 (7·4%)	
Disease duration (years)		2·3 (0·7 - 9·1)	2·6 (0·7 - 10·0)	0·56
Age at diagnosis (years)		22·7 (15·3 - 32·7)	24·5 (16·8 - 38·6)	0·014
Montreal location	L1	158 (23·5%)	68 (34·0%)	0·027
	L2	177 (26·4%)	42 (21·0%)	
	L3	330 (49·2%)	89 (44·5%)	
	L4	6 (0·9%)	1 (0·5%)	
Montreal L4		90 (13·4%)	18 (9·0%)	0·11
Montreal behaviour	B1	407 (60·6%)	130 (66·0%)	0·40
	B2	187 (27·8%)	48 (24·4%)	
	B3	78 (11·6%)	19 (9·6%)	
Perianal		97 (14·4%)	36 (17·8%)	0·26
Immunomodulator	azathioprine	325 (48·4%)	95 (47·0%)	0·56
	mercaptopurine	55 (8·2%)	14 (6·9%)	
	methotrexate	36 (5·4%)	16 (7·9%)	
	none	256 (38·1%)	77 (38·1%)	
Steroids		207 (30·7%)	52 (25·7%)	0·19
Previous resectional surgery		150 (22·3%)	41 (20·3%)	0·63
HBI		5 (3 - 9)	6 (4 - 9)	0·33
sPCDAI		25 (15 - 50)	35 (11 - 49)	0·99
BMI		22·5 (19·5 - 26·8)	22·9 (20·1 - 27·3)	0·46
Hemoglobin (g/L)		125 (114 - 135)	124 (116 - 135)	0·86
White cell count ($\times 10^9$/L)		7·9 (6·1 - 10·4)	8·1 (6·2 - 10·5)	0·56
Platelet count ($\times 10^9$/L)		342 (285 - 411)	352 (282 - 442)	0·28
Albumin (g/L)		38 (34 - 42)	39 (35 - 42)	0·29
CRP (mg/L)		9 (3 - 24)	9 (3 - 24)	0·52
Fecal calprotectin (µg/g)		431 (164 - 872)	380 (133 - 722)	0·40

Patients who switched from Remicade to CT-P13 in the first year of the study are excluded. Medians and interquartile range (IQR) are presented unless otherwise stated. The significance of differences between continuous variables was calculated using the Mann-Whitney U test. Differences between categorical variables were sought using Fisher's exact test was used.

10.3 Discussion of paper

The PANTS study represents the largest clinical dataset I studied during my thesis. The published data in this chapter represent analyses of the first year only, since some patients will not complete final follow-up until later in 2020. In chapter 11, I will detail other analyses that have been completed and that are planned. However, even the first year's data have provided useful insights into management of Crohn's disease. They highlight the importance of good early anti-TNF drug concentrations and provide further understanding of the relationship between active inflammation, drug concentrations and immunogenicity.

Since the publication of the PANTS study in 2019, it has been cited 65 times. One of the principal areas of interest in the paper is the contribution to the literature on proactive therapeutic drug monitoring. PANTS provides strong evidence of an association between low drug levels and poorer outcomes, but as an uncontrolled clinical study cannot demonstrate that intervening to push drug levels higher would change outcome. Assa *et al.* recently published the results of the PAILOT trial which randomised children with Crohn's disease to proactive or reactive therapeutic drug monitoring soon after initiation of adalimumab.[149] They showed that dose adjustment proactive drug monitoring resulted in higher corticosteroid-free remission.

With respect to the impact of thiopurines on immunogenicity and loss of response, Roblin *et al.* have published data from a trial where patients were randomised to receive a thiopurine or not when starting a second anti-TNF following immune-mediated failure of the first.[150] Combination therapy was associated with lower rates of clinical failure and more favourable pharmacokinetics.

The data from PANTS have also been used to inform clinical guidelines in IBD[151,152] as well as reviews of the importance of therapeutic drug monitoring and immunogenicity in other disease areas.[153]

11 Conclusions, implications and future research

11.1 Summary of thesis and conclusions

The last twenty years have seen great advances in our understanding of the pathogenesis, diagnosis and treatment of Crohn's disease. We now think that Crohn's disease results from complex interactions between genetics, environmental factors, the intestinal microbiome and the immune system.[19] However, many of the details still need to be elucidated.

In the first section of this thesis, I explorer the interactions between host genetics and the intestinal microbiome. Using faecal samples from both patients with Crohn's disease and from volunteers without gastrointestinal disease, I was able to show differences in faecal microbiota when stratified by the presence of disease, but not when stratified by *NOD2* genotype. This finding differs from those reported by some other groups[154]. Strengths of the analyses presented here include the high proportion of individuals who carried two Crohn's-associated *NOD2* mutations, since individuals were recruited on the basis of known genotype. However, it may be that a genotype-microbiota association is only evident in the presence of active disease, or that the effect is tissue-specific and only seen in the terminal ileal mucosal-associated microbiota.

Early diagnosis of Crohn's disease is important, since delay in diagnosis can lead to an increased rate of complications.[46] Although definitive diagnosis requires endoscopic and histological assessment, non-invasive biomarkers are important to reduce the number of negative colonoscopies performed. In chapter 6, I presented data from the use of faecal calprotectin a large secondary care referral cohort. I demonstrated a high area under the receiving operating characteristic curve of 0.97. The combination of faecal calprotectin and alarm symptoms had 100% sensitivity for the diagnosis of Crohn's disease. Most previous

studies had either used people with an established diagnosis of inflammatory bowel disease or had small sample size.[155–160] The data in chapter 6 therefore provide further strong evidence for the routine use of faecal calprotectin as part of the diagnostic pathway for Crohn's disease.

Faecal calprotectin has also been increasingly used to monitor patients with known Crohn's disease. In chapter 7, I demonstrated that elevated faecal calprotectin, independent of symptoms, is associated with an increased risk of developing stricturing or penetrating disease, hospital admission or surgery, both as individual endpoints and as a composite endpoint. This effect was seen in patients with ileal, colonic or ileocolonic disease. These data, alongside others such as the recent CALM study[161], support the concept that patients should be treated with a goal of healing the mucosa, rather than simply to alleviate symptoms.

Nonetheless, all of the treatments currently used to maintain remission of Crohn's disease carry some risk of long-term adverse effects, including infection and cancer.[162–164] Although biosimilars have led to marked reductions in price, the cost of medicines remains a significant concern for health systems, and in countries with co-payments for medication, the patients themselves. NICE recommend review of discontinuation of biologics for Crohn's at 12 month intervals,[87,100,108] but evidence to guide these decisions is still needed. In chapters 8 and 9, I have presented data useful to guide clinicians and patients when considering withdrawal. These data can be interpreted in the light of a patient's individual circumstances. For some, such as a student facing important university examinations the following year, a greater than 50% chance of relapse within two years may be too high. For others, the chance of having time off medication is worth taking, and they can be reassured

by the high chance of successful retreatment. The predictive factors identified in both cases are also useful when stratifying individual patient's risk.

Finally, in chapter 10, I have presented, to the best of my knowledge, the largest prospective study to date of anti-TNF therapy for the treatment of Crohn's. I have demonstrated the importance of achieving good drug levels, and the negative impact of immunogenicity, particularly for infliximab. Although several baseline factors were associated with negative outcomes, including older age, smoking and non-use of immunomodulators, accurate prediction of drug response remains difficult, even with the breadth and depth of data available here.

11.2 Ongoing and future work

All the work presented here has already led onto further projects with which I am engaged. With respect, to the microbiome work, I was a co-applicant for the PREdiCCt study, a successfully-funded 3100-patient study looking at understanding which dietary, microbial, genetic and environmental factors are associated with an increased risk of relapse in Crohn's disease. Recruitment is underway around the UK, and I regularly participate in meetings of the analytical committee. I am also actively engaged with the IBD BioResource, a recallable cohort of patients funded by the MRC. As part of this, we are recruiting 1000 patients at the time of diagnosis, and sampling includes faecal samples to look at differences in gut microbiota.

With regards to calprotectin, most published data including that in chapter 6 focuses on patients after referral to secondary care. I recently helped analyse a cohort of 789 patients presenting with lower gastrointestinal symptoms to primary care as part of a pilot of permitting GPs to test calprotectin prior to making a referral to gastroenterology.[57] I am

now working with colleagues to extend these analyses to children and to explore the impact of the introduction of calprotectin testing on diagnostic delay.

The anti-TNF withdrawal work has been included in an individual patient-level meta-analysis which has been presented as an abstract,[165] and there are plans to write a full paper. These data were also considered during the planning of the BIOCYCLE EU-Horizon 2020-funded project (https://biocycle-project.eu/). This includes SPARE, an international randomised controlled trial of thiopurine withdrawal, anti-TNF withdrawal or neither in patients on stable combination therapy. SPARE has completed recruitment and is in the follow-up phase at present.

The PANTS study completed recruitment in July 2016, so the final patient will reach three years in July 2019. I am currently working on analysis of the year 2 and 3 data, including more detailed analysis of loss of response, outcomes of treatment of loss of response and quality of life outcomes. We collected longitudinal samples from patients in the PANTS cohort that included DNA, RNA, serum and faecal samples. I worked with colleagues from Sanger to analyse the genetics of immunogenicity; this has now been published.[166–169] We have described a genome-wide significant association between HLA DQA1*05 and the development of immunogenicity for both infliximab and adalimumab. More work is needed to fully understand the clinical implications of this finding, but it may permit more informed choices of therapy in future.

Colleagues in Genos, Croatia have used HPLC to generate IgG glycans data from the baseline samples for most of the PANTS cohort, and from week 14 for a subset. I am now working with others to see whether there are any important clinical or biological associations between changes in IgG glycosylation between those who respond well and

those do not. We are now awaiting serum proteomic data, measured using Olink's proximity extension assay (https://www.olink.com), DNA methylation data measured using Illumina EPIC human methylation array and gene transcription measured using RNA sequencing. I will be working with colleagues in the UK and internationally to analyse and integrate these datasets into a multi-omic model of anti-TNF response, non-response and immunogenicity.

Beyond PANTS, I am in the early planning stages of a pragmatic randomised controlled trial, aiming to extend what I have learned from this large non-interventional study to one with multiple treatment arms. This should help simultaneously answer the question of which treatment is more effective overall, as well as offering opportunities to see whether decisions between therapies can be better informed at baseline.

Collectively, these projects all further our understanding of Crohn's disease treatment and move us closer to the goal of personalising treatment for the individual patient.

References (not including references within published papers)

1. Kalla R, Ventham NT, Satsangi J, Arnott IDR. Crohn's disease. BMJ 2014;349:g6670.

2. Dalziel TK. Chronic interstitial enteritis. Br Med J 1913;2:1068–70.

3. Crohn BB, Ginzburg L, Oppenheimer GD. Regional ileitis: a pathological clinical entity. JAMA 1932;99:1323–132.

4. Henderson P, Hansen R, Cameron FL, Gerasimidis K, Rogers P, Bisset MW, et al. Rising incidence of pediatric inflammatory bowel disease in Scotland*. Inflamm Bowel Dis 2012;18:999–1005.

5. Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 2012;142:46-54.e42; quiz e30.

6. Ng SC. Epidemiology of inflammatory bowel disease: Focus on Asia. Best Pract Res Clin Gastroenterol 2014;28:363–72.

7. Tysk C, Lindberg E, Järnerot G, Flodérus-Myrhed B, Jarnerot G, Floderus-Myrhed B, et al. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut 1988;29:990–6.

8. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than thirty distinct susceptibility loci for Crohn's disease. Nat Genet 2009;40:955–62.

9. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119–24.

10. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet 2015;47:979–86.

11. Luo Y, De Lange KM, Jostins L, Moutsianas L, Randall J, Kennedy NA, et al. Exploring the genetic architecture of inflammatory bowel disease by whole-genome sequencing identifies association at ADCY7. Nat Genet 2017;49:186–92.

12. de Lange KM, Moutsianas L, Lee JC, Lamb CA, Luo Y, Kennedy NA, et al. Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. Nat Genet 2017;49:256–61.

13. Philpott DJ, Sorbara MT, Robertson SJ, Croitoru K, Girardin SE. NOD proteins: regulators of inflammation in health and disease. Nat Rev Immunol 2014;14:9–23.

14. Mukherjee T, Hovingh ES, Foerster EG, Abdel-Nour M, Philpott DJ, Girardin SE. NOD1 and NOD2 in inflammation, immunity and disease. Arch. Biochem. Biophys.2019;670:69–81.

15. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nuñez G, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005;307:731–4.

16. Huang H, Fang M, Jostins L, Umićević Mirkov M, Boucher G, Anderson CA, et al. Fine-mapping inflammatory bowel disease loci to single-variant resolution. Nature 2017;547:173–8.

17. Khera A V., Chaffin M, Aragam KG, Haas ME, Roselli C, Choi SH, et al. Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. Nat. Genet.2018;50:1219–24.

18. Cleynen I, Boucher G, Jostins L, Schumm LPP, Zeissig S, Ahmad T, et al. Inherited determinants of Crohn's disease and ulcerative colitis phenotypes: A genetic association study. Lancet 2016;387:156–67.

19. Sartor RB. Genetics and environmental interactions shape the intestinal microbiome to promote inflammatory bowel disease versus mucosal homeostasis. Gastroenterology 2010;139:1816–9.

20. Hold GL, Smith M, Grange C, Watt ER, El-Omar EM, Mukhopadhya I. Role of the gut microbiota in inflammatory bowel disease pathogenesis: what have we learnt in the past 10 years? World J Gastroenterol 2014;20:1192–210.

21. Nguyen HTT, Dalmasso G, Müller S, Carrière J, Seibold F, Darfeuille-Michaud A. Crohn's disease-associated adherent invasive *Escherichia coli* modulate levels of microRNAs in intestinal epithelial cells to reduce autophagy. Gastroenterology 2014;146:508–19.

22. Claesson MJ, Clooney AG, O'Toole PW. A clinician's guide to microbiome analysis. Nat Rev Gastroenterol Hepatol 2017; Epub ahead of print Aug 9, 2017. Available from doi:10.1038/nrgastro.2017.97.

23. Di Bella JM, Bao Y, Gloor GB, Burton JP, Reid G. High throughput sequencing methods and analysis for microbiome research. J Microbiol Methods 2013;95:401–14.

24. Gerasimidis K, Bertz M, Quince C, Brunner K, Bruce A, Combet E, et al. The effect of DNA extraction methodology on gut microbiota research applications. BMC Res Notes 2016;9:365.

25. Cruaud P, Vigneron A, Lucchetti-Miganeh C, Ciron PE, Godfroy A, Cambon-Bonavita M-A. Influence of DNA extraction method, 16S rRNA targeted hypervariable regions, and sample origin on microbial diversity detected by 454 pyrosequencing in marine chemosynthetic ecosystems. Appl Environ Microbiol 2014;80:4626–39.

26. Teng F, Darveekaran Nair SS, Zhu P, Li S, Huang S, Li X, et al. Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. Sci Rep 2018;8:16321.

27. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet 2017;390:2769–78.

28. van der Sloot KWJ, Amini M, Peters V, Dijkstra G, Alizadeh BZ. Inflammatory Bowel Diseases: Review of Known Environmental Protective and Risk Factors Involved. Inflamm Bowel Dis 2017;23:1499–509.

29. Burke KE, Boumitri C, Ananthakrishnan AN. Modifiable Environmental Factors in Inflammatory Bowel Disease. Curr Gastroenterol Rep 2017;19:21.

30. Berkowitz L, Schultz BM, Salazar GA, Pardo-Roa C, Sebastián VP, Álvarez-Lobos MM, et al. Impact of Cigarette Smoking on the Gastrointestinal Tract Inflammation: Opposing Effects in Crohn's Disease and Ulcerative Colitis. Front Immunol 2018;9. Epub ahead of print Jan 30, 2018. Available from doi:10.3389/fimmu.2018.00074.

31. Parkes GC, Whelan K, Lindsay JO. Smoking in inflammatory bowel disease: Impact on disease course and insights into the aetiology of its effect. J Crohns Colitis 2014; Epub ahead of print Mar 10, 2014. Available from doi:10.1016/j.crohns.2014.02.002.

32. Mahid SS, Minor KS, Soto RE, Hornung CA, Galandiuk S. Smoking and inflammatory bowel disease: a meta-analysis. Mayo Clin Proc 2006;81:1462–71.

33. Burisch J, Pedersen N, Cukovic-Cavka S, Turk N, Kaimakliotis I, Duricova D, et al. Environmental factors in a population-based inception cohort of inflammatory bowel disease patients in Europe — An ECCO-EpiCom study. J Crohn's Colitis 2014;8:607–16.

34. Lunney PC, Kariyawasam VC, Wang RR, Middleton KL, Huang T, Selinger CP, et al. Smoking prevalence and its influence on disease course and surgery in Crohn's disease and ulcerative colitis. Aliment Pharmacol Ther 2015;42:61–70.

35. Chassaing B, Koren O, Goodrich JK, Poole AC, Srinivasan S, Ley RE, et al. Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. Nature 2015;519:92–6.

36. Ventham NT, Kennedy NA, Nimmo ER, Satsangi J. Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. Gastroenterology

2013;145:293–308.

37. Adams AT, Kennedy NA, Hansen R, Ventham NT, O'Leary KR, Drummond HE, et al. Two-stage genome-wide methylation profiling in childhood-onset Crohn's Disease implicates epigenetic alterations at the VMP1/MIR21 and HLA loci. Inflamm Bowel Dis 2014;20:1784–93.

38. Ventham NT, Kennedy NA, Adams AT, Kalla R, Heath S, O'Leary KR, et al. Integrative epigenome-wide analysis demonstrates that DNA methylation may mediate genetic risk in inflammatory bowel disease. Nat Commun 2016;7:13507.

39. Nimmo ER, Prendergast JG, Aldhous MC, Kennedy NA, Henderson P, Drummond HE, et al. Genome-wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway. Inflamm Bowel Dis 2012;18:889–99.

40. Jenke AC, Zilbauer M. Epigenetics in inflammatory bowel disease. Curr Opin Gastroenterol 2012;28:577–84.

41. Klein K, Gay S. Epigenetics in rheumatoid arthritis. Curr Opin Rheumatol 2015;27:76–82.

42. Hedrich CM. Epigenetics in SLE. Curr Rheumatol Rep 2017;19:58.

43. Kalla R, Ventham N., Kennedy N., Quintana JF, Nimmo ER, Buck AH, et al. MicroRNAs: new players in IBD. Gut 2015;64:504–13.

44. Jones G-R, Lyons M, Plevris N, Jenkinson PW, Bisset C, Burgess C, et al. IBD prevalence in Lothian, Scotland, derived by capture-recapture methodology. Gut

2019;68:1953–60.

45. Torres J, Mehandru S, Colombel J-F, Peyrin-Biroulet L. Crohn's disease. Lancet 2017;389:1741–55.

46. Schoepfer AM, Dehlavi M-A, Fournier N, Safroneeva E, Straumann A, Pittet V, et al. Diagnostic delay in Crohn's disease is associated with a complicated disease course and increased operation rate. Am J Gastroenterol 2013;108:1744–53; quiz 1754.

47. Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin Gastroenterol Hepatol 2012;10:712-721.e4.

48. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl 1989;170:2–6; discussion 16-9.

49. Røseth AG, Fagerhol MK, Aadland E, Schjønsby H. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. Scand J Gastroenterol 1992;27:793–8.

50. D'Haens G, Ferrante M, Vermeire S, Baert F, Noman M, Moortgat L, et al. Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. Inflamm Bowel Dis 2012;18:2218–24.

51. Schoepfer AM, Beglinger C, Straumann A, Trummler M, Renzulli P, Seibold F. Ulcerative colitis: correlation of the Rachmilewitz endoscopic activity index with fecal calprotectin, clinical activity, C-reactive protein, and blood leukocytes. Inflamm Bowel Dis 2009;15:1851–8.

52. Schoepfer AM, Beglinger C, Straumann A, Trummler M, Vavricka SR, Bruegger

LE, et al. Fecal calprotectin correlates more closely with the Simple Endoscopic Score for Crohn's disease (SES-CD) than CRP, blood leukocytes, and the CDAI. Am J Gastroenterol 2010;105:162–9.

53. Sipponen T, Savilahti E, Kolho K-L, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. Inflamm Bowel Dis 2008;14:40–6.

54. Sipponen T, Kärkkäinen P, Savilahti E, Kolho K-L, Nuutinen H, Turunen U, et al. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. Aliment Pharmacol Ther 2008;28:1221–9.

55. Kennedy NA, Clark A, Walkden A, Chang JCW, Fascí-Spurio F, Muscat M, et al. Clinical utility and diagnostic accuracy of faecal calprotectin for IBD at first presentation to gastroenterology services in adults aged 16-50 years. J Crohns Colitis 2015;9:41–9.

56. Henderson P, Casey A, Lawrence SJ, Kennedy NA, Kingstone K, Rogers P, et al. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease. Am J Gastroenterol 2012;107:941–9.

57. Walker GJ, Moore L, Heerasing N, Hendy P, Perry MH, Mcdonald TJ, et al. Faecal calprotectin effectively excludes inflammatory bowel disease in 789 symptomatic young adults with/without alarm symptoms: A prospective UK primary care cohort study. Aliment Pharmacol Ther 2018;47:1103–16.

58. van Rheenen PF, Van de Vijver E, Fidler V. Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis. Bmj 2010;341:c3369–c3369.

59. Chang S, Malter L, Hudesman D. Disease monitoring in inflammatory bowel disease. World J Gastroenterol 2015;21:11246–59.

60. D'Iincà R, Caccaro R. Measuring disease activity in Crohn's disease: What is currently available to the clinician. Clin Exp Gastroenterol 2014;7:151–61.

61. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 2005;19 Suppl A:5–36.

62. Cosnes J, Gower-Rousseau C, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterology 2011;140:1785-1794.e4.

63. Dziechciarz P, Horvath A, Shamir R, Szajewska H. Meta-analysis: enteral nutrition in active Crohn's disease in children. Aliment Pharmacol Ther 2007;26:795–806.

64. Rigaud D, Cosnes J, Le Quintrec Y, René E, Gendre JP, Mignon M. Controlled trial comparing two types of enteral nutrition in treatment of active Crohn's disease: elemental versus polymeric diet. Gut 1991;32:1492–7.

65. Royall D, Jeejeebhoy KN, Baker JP, Allard JP, Habal FM, Cunnane SC, et al. Comparison of amino acid v peptide based enteral diets in active Crohn's

disease: clinical and nutritional outcome. Gut 1994;35:783–7.

66. Wall CL, Gearry RB, Day AS. Treatment of Active Crohn's Disease with Exclusive and Partial Enteral Nutrition: A Pilot Study in Adults. Inflamm Intest Dis 2018;2:219–27.

67. Lee J, Allen R, Ashley S, Becker S, Cummins P, Gbadamosi A, et al. British Dietetic Association evidence-based guidelines for the dietary management of Crohn's disease in adults. J Hum Nutr Diet 2014;27:207–18.

68. Heerasing N, Thompson B, Hendy P, Heap GA, Walker G, Bethune R, et al. Exclusive enteral nutrition provides an effective bridge to safer interval elective surgery for adults with Crohn's disease. Aliment Pharmacol Ther 2017;45:660–9.

69. Levine A, Wine E, Assa A, Sigall Boneh R, Shaoul R, Kori M, et al. Crohn's Disease Exclusion Diet Plus Partial Enteral Nutrition Induces Sustained Remission in a Randomized Controlled Trial. Gastroenterology 2019;157:440-450.e8.

70. Svolos V, Hansen R, Nichols B, Quince C, Ijaz UZ, Papadopoulou RT, et al. Treatment of Active Crohn's Disease With an Ordinary Food-based Diet That Replicates Exclusive Enteral Nutrition. Gastroenterology 2019;156:1354-1367.e6.

71. Sigall-Boneh R, Pfeffer-Gik T, Segal I, Zangen T, Boaz M, Levine A. Partial enteral nutrition with a Crohn's disease exclusion diet is effective for induction of remission in children and young adults with Crohn's disease. Inflamm Bowel Dis 2014;20:1353–60.

72. Rezaie A, Kuenzig ME, Benchimol EI, Griffiths AM, Otley AR, Steinhart AH, et al. Budesonide for induction of remission in Crohn's disease. Cochrane database Syst Rev 2015;CD000296.

73. Benchimol EI, Seow CH, Steinhart AH, Griffiths AM. Traditional corticosteroids for induction of remission in Crohn's disease. Cochrane database Syst Rev 2008;CD006792.

74. Gomollón F, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, et al. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. J Crohn's Colitis 2017;11:3–25.

75. Selinger CP, Parkes GC, Bassi A, Limdi JK, Ludlow H, Patel P, et al. Assessment of steroid use as a key performance indicator in inflammatory bowel disease—analysis of data from 2385 UK patients. Aliment Pharmacol Ther 2019;50:1009–18.

76. Bean RH. The treatment of chronic ulcerative colitis with 6-mercaptopurine. Med J Aust 1962;49(2):592–3.

77. Rhodes J, Bainton D, Beck P, Campbell H. Controlled trial of azathioprine in Crohn's disease. Lancet (London, England) 1971;2:1273–6.

78. de Boer NKH, van Bodegraven A a, Jharap B, de Graaf P, Mulder CJJ. Drug Insight: pharmacology and toxicity of thiopurine therapy in patients with IBD. Nat Clin Pract Gastroenterol Hepatol 2007;4:686–94.

79. Chande N, Patton PH, Tsoulis DJ, Thomas BS, MacDonald JK. Azathioprine or 6-

mercaptopurine for maintenance of remission in Crohn's disease. Cochrane Database Syst Rev 2015;CD000067.

80. Chande N, Townsend CM, Parker CE, MacDonald JK. Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease. Cochrane Database Syst Rev 2016;10:CD000545.

81. Rampton DS. Methotrexate in Crohn's disease. Gut 2001;48:790–1.

82. Patel V, Wang Y, MacDonald JK, McDonald JW, Chande N. Methotrexate for maintenance of remission in Crohn's disease. Cochrane Database Syst Rev 2014;CD006884.

83. Monaco C, Nanchahal J, Taylor P, Feldmann M. Anti-TNF therapy: past, present and future. Int Immunol 2015;27:55–62.

84. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 2002;359:1541–9.

85. Hanauer SB, Sandborn WJ, Rutgeerts P, Fedorak RN, Lukas M, MacIntosh D, et al. Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial. Gastroenterology 2006;130:323–33; quiz 591.

86. Colombel JF, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, et al. Adalimumab for Maintenance of Clinical Response and Remission in Patients With Crohn's Disease: The CHARM Trial. Gastroenterology 2007;132:52–65.

87. NICE. Infliximab and adalimumab for the treatment of Crohn's disease. NICE

Guid 2010;

88. Colombel JF, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010;362:1383–95.

89. Chalhoub JM, Rimmani HH, Gumaste V V., Sharara AI. Systematic Review and Meta-analysis: Adalimumab Monotherapy Versus Combination Therapy with Immunomodulators for Induction and Maintenance of Remission and Response in Patients with Crohn's Disease. Inflamm Bowel Dis 2017;23:1316–27.

90. D'Haens G, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, et al. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. Lancet (London, England) 2008;371:660–7.

91. Hoekman DR, Stibbe JA, Baert FJ, Caenepeel P, Vergauwe P, De Vos M, et al. Long-term Outcome of Early Combined Immunosuppression Versus Conventional Management in Newly Diagnosed Crohn's Disease. J Crohn's Colitis 2018;12:517–24.

92. Vermeire S, Gils A, Accossato P, Lula S, Marren A. Immunogenicity of biologics in inflammatory bowel disease. Therap Adv Gastroenterol 2018;11:1756283X17750355.

93. Ishii-Watabe A, Kuwabara T. Biosimilarity assessment of biosimilar therapeutic monoclonal antibodies. Drug Metab Pharmacokinet 2019;34:64–70.

94. Park W, Hrycaj P, Jeka S, Kovalenko V, Lysenko G, Miranda P, et al. A randomised,

double-blind, multicentre, parallel-group, prospective study comparing the pharmacokinetics, safety, and efficacy of CT-P13 and innovator infliximab in patients with ankylosing spondylitis: the PLANETAS study. Ann Rheum Dis 2013;72:1605–12.

95. Yoo DH, Hrycaj P, Miranda P, Ramiterre E, Piotrowski M, Shevchuk S, et al. A randomised, double-blind, parallel-group study to demonstrate equivalence in efficacy and safety of CT-P13 compared with innovator infliximab when coadministered with methotrexate in patients with active rheumatoid arthritis: the PLANETRA study. Ann Rheum Dis 2013;72:1613–20.

96. Bloomgren G, Richman S, Hotermans C, Subramanyam M, Goelz S, Natarajan A, et al. Risk of Natalizumab-Associated Progressive Multifocal Leukoencephalopathy. N Engl J Med 2012;366:1870–80.

97. Panés J, Salas A. Past, Present and Future of Therapeutic Interventions Targeting Leukocyte Trafficking in Inflammatory Bowel Disease. J Crohn's Colitis 2018;12:S633–40.

98. Parikh A, Stephens K, Major E, Fox I, Milch C, Sankoh S, et al. A Programme for Risk Assessment and Minimisation of Progressive Multifocal Leukoencephalopathy Developed for Vedolizumab Clinical Trials. Drug Saf 2018;41:807–16.

99. Sandborn WJ, Feagan BG, Rutgeerts P, Hanauer S, Colombel J-F, Sands BE, et al. Vedolizumab as Induction and Maintenance Therapy for Crohn's Disease. N Engl J Med 2013;369:711–21.

100. NICE. TA352 Vedolizumab for treating moderately to severely active Crohn's

disease after prior therapy [Internet]. 2014 [cited 2019 Apr 4];Available from: https://www.nice.org.uk/guidance/ta352

101. Samaan MA, Pavlidis P, Johnston E, Warner B, Digby-Bell J, Koumoutsos I, et al. Vedolizumab: early experience and medium-term outcomes from two UK tertiary IBD centres. Frontline Gastroenterol 2017;8:196–202.

102. Plevris N, Chuah CS, Allen RM, Arnott ID, Brennan PN, Chaudhary S, et al. Real-world effectiveness and safety of Vedolizumab for the treatment of Inflammatory Bowel Disease: The Scottish Vedolizumab Cohort. J Crohns Colitis 2019; Epub ahead of print Feb 14, 2019. Available from doi:10.1093/ecco-jcc/jjz042.

103. Lenti MV, Levison S, Eliadou E, Willert R, Kemp K, Carter A, et al. A real-world, long-term experience on effectiveness and safety of vedolizumab in adult patients with inflammatory bowel disease: The Cross Pennine study. Dig Liver Dis 2018;50:1299–304.

104. Cummings F, Gaya DR, Levison S, Subramanian S, Owen G, Rathmell A, et al. A retrospective observational study of early experiences of vedolizumab treatment for inflammatory bowel disease in the UK: The REVIVE study. Medicine (Baltimore) 2019;98:e14681.

105. Sands BE, Peyrin-Biroulet L, Loftus E V., Danese S, Colombel JF, Törüner M, et al. Vedolizumab versus Adalimumab for moderate-to-severe ulcerative colitis. N Engl J Med 2019;381:1215–26.

106. Teng MWL, Bowman EP, McElwee JJ, Smyth MJ, Casanova J-L, Cooper AM, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-

mediated inflammatory diseases. Nat Med 2015;21:719–29.

107. Feagan BG, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, et al. Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. N Engl J Med 2016;375:1946–60.

108. NICE. TA456 Ustekinumab for moderately to severely active Crohn's disease after previous treatment [Internet]. 2017 [cited 2019 Apr 4];Available from: https://www.nice.org.uk/guidance/TA456

109. Pérez-Jeldres T, Tyler CJ, Boyer JD, Karuppuchamy T, Yarur A, Giles DA, et al. Targeting Cytokine Signaling and Lymphocyte Traffic via Small Molecules in Inflammatory Bowel Disease: JAK Inhibitors and S1PR Agonists. Front Pharmacol 2019;10:212.

110. ClinicalTrials.gov. A Long-term Active Treatment Study of Mongersen (GED-0301) in Subjects With Crohn's Disease [Internet]. 2019 [cited 2019 Apr 4];Available from: https://clinicaltrials.gov/ct2/show/NCT02641392

111. Feagan BG, Sandborn WJ, D'Haens G, Lee SD, Allez M, Fedorak RN, et al. Randomised clinical trial: Vercirnon, an oral CCR9 antagonist, vs. placebo as induction therapy in active Crohn's disease. Aliment Pharmacol Ther 2015;42:1170–81.

112. Peyrin-Biroulet L, Harmsen WS, Tremaine WJ, Zinsmeister AR, Sandborn WJ, Loftus E V, et al. Surgery in a population-based cohort of Crohn's disease from Olmsted County, Minnesota (1970-2004). Am J Gastroenterol 2012;107:1693–701.

113. Ahmad A, Laverty AA, Alexakis C, Cowling T, Saxena S, Majeed A, et al. Changing nationwide trends in endoscopic, medical and surgical admissions for inflammatory bowel disease: 2003–2013. BMJ Open Gastroenterol 2018;5:e000191.

114. Holder-Murray J, Marsicovetere P, Holubar SD. Minimally Invasive Surgery for Inflammatory Bowel Disease. Inflamm Bowel Dis 2015;21:1.

115. Ponsioen CY, de Groof EJ, Eshuis EJ, Gardenbroek TJ, Bossuyt PMM, Hart A, et al. Laparoscopic ileocaecal resection versus infliximab for terminal ileitis in Crohn's disease: a randomised controlled, open-label, multicentre trial. Lancet Gastroenterol Hepatol 2017;2:785–92.

116. Pariente B, Cosnes J, Danese S, Sandborn WJ, Lewin M, Fletcher JG, et al. Development of the Crohn's disease digestive damage score, the Lémann score. Inflamm Bowel Dis 2011;17:1415–22.

117. Cosnes J, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, et al. Long-Term Evolution of Disease Behavior of Crohn's Disease. Inflamm Bowel Dis 2002;8:244–50.

118. Solberg IC, Vatn MH, Høie O, Stray N, Sauar J, Jahnsen J, et al. Clinical Course in Crohn's Disease: Results of a Norwegian Population-Based Ten-Year Follow-Up Study. Clin Gastroenterol Hepatol 2007;5:1430–8.

119. Cosnes J, Bourrier A, Nion-Larmurier I, Sokol H, Beaugerie L, Seksik P. Factors affecting outcomes in Crohn's disease over 15 years. Gut 2012;61:1140–5.

120. Peyrin-Biroulet L, Sandborn W, Sands BE, Reinisch W, Bemelman W, Bryant R V,

et al. Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE): Determining Therapeutic Goals for Treat-to-Target. Am J Gastroenterol 2015;110:1324–38.

121. Jones GR, Kennedy NA, Lees CW, Arnott ID, Satsangi J. Systematic review: The use of thiopurines or anti-TNF in post-operative Crohn's disease maintenance--progress and prospects. Aliment Pharmacol Ther 2014;39:1253–65.

122. De Cruz P, Kamm MA, Hamilton AL, Ritchie KJ, Krejany EO, Gorelik A, et al. Crohn's disease management after intestinal resection: A randomised trial. Lancet 2015;385:1406–17.

123. Thadhani R, Tonelli M. Cohort Studies: Marching Forward. Clin J Am Soc Nephrol 2006;1:1117–23.

124. Euser AM, Zoccali C, Jager KJ, Dekker FW. Cohort Studies: Prospective versus Retrospective. Nephron Clin Pract 2009;113:c214–7.

125. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the Areas under Two or More Correlated Receiver Operating Characteristic Curves: A Nonparametric Approach. Biometrics 1988;44:837.

126. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:77.

127. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577–94.

128. Costea PI, Zeller G, Sunagawa S, Pelletier E, Alberti A, Levenez F, et al. Towards

standards for human fecal sample processing in metagenomic studies. Nat Biotechnol 2017;35:1069–76.

129. Videnska P, Smerkova K, Zwinsova B, Popovici V, Micenkova L, Sedlar K, et al. Stool sampling and DNA isolation kits affect DNA quality and bacterial composition following 16S rRNA gene sequencing using MiSeq Illumina platform. Sci Rep 2019;9:1–14.

130. Marques FZ, Jama HA, Tsyganov K, Gill PA, Rhys-Jones D, Muralitharan RR, et al. Guidelines for Transparency on Gut Microbiome Studies in Essential and Experimental Hypertension. Hypertension 2019;74:1279–93.

131. Pollock J, Glendinning L, Wisedchanwet T, Watson M. The madness of microbiome: Attempting to find consensus "best practice" for 16S microbiome studies. Appl. Environ. Microbiol.2018;84. Epub ahead of print Apr 1, 2018. Available from doi:10.1128/AEM.02627-17.

132. Assenmacher DM, Fields SD, Crupper SS. Comparison of Commercial Kits for Recovery and Analysis of Bacterial DNA From Fingerprints. J Forensic Sci 2020;1556-4029.14280.

133. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe 2014;15:382–92.

134. Chen J-J, Fan P-C, Kou G, Chang S-W, Chen Y-T, Lee C-C, et al. Meta-Analysis: Urinary Calprotectin for Discrimination of Intrinsic and Prerenal Acute Kidney Injury. J Clin Med 2019;8. Epub ahead of print Jan 10, 2019. Available from doi:10.3390/jcm8010074.

135. Muhlebach MS, Clancy JP, Heltshe SL, Ziady A, Kelley T, Accurso F, et al. Biomarkers for cystic fibrosis drug development. J Cyst Fibros 2016;15:714–23.

136. Ometto F, Friso L, Astorri D, Botsios C, Raffeiner B, Punzi L, et al. Calprotectin in rheumatic diseases. Exp Biol Med (Maywood) 2017;242:859–73.

137. Lewis JD. The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. Gastroenterology 2011;140:1817–26.

138. Sands BE, Arsenault JE, Rosen MJ, Alsahli M, Bailen L, Banks P, et al. Risk of early surgery for Crohn's disease: implications for early treatment strategies. Am J Gastroenterol 2003;98:2712–8.

139. Yui S, Nakatani Y, Mikami M. Calprotectin (S100A8/S100A9), an Inflammatory Protein Complex from Neutrophils with a Broad Apoptosis-Inducing Activity. Biol Pharm Bull 2003;26:753–60.

140. Ross KF, Herzberg MC. Calprotectin Expression by Gingival Epithelial Cells. Infect Immun 2001;69:3248–54.

141. Wang S, Song R, Wang Z, Jing Z, Wang S, Ma J. S100A8/A9 in inflammation. Front. Immunol.2018;9:1298.

142. Coveney AP, Wang W, Kelly J, Liu JH, Blankson S, Wu Q Di, et al. Myeloid-related protein 8 induces self-tolerance and cross-tolerance to bacterial infection via TLR4- and TLR2-mediated signal pathways. Sci Rep 2015;5:13694.

143. Gray RD, Imrie M, Boyd AC, Porteous D, Innes JA, Greening AP. Sputum and serum calprotectin are useful biomarkers during CF exacerbation. J Cyst Fibros 2010;9:193–8.

144. Chmielewski PP, Strzelec B. Elevated leukocyte count as a harbinger of systemic inflammation, disease progression, and poor prognosis: A review. Folia Morphol.2018;77:171–8.

145. Gu Y, Hu K, Huang Y, Zhang Q, Liu L, Meng G, et al. White blood cells count as an indicator to identify whether obesity leads to increased risk of type 2 diabetes. Diabetes Res Clin Pract 2018;141:140–7.

146. Torres J, Boyapati RK, Kennedy NA, Louis E, Colombel J-F, Satsangi J. Systematic Review of Effects of Withdrawal of Immunomodulators or Biologic Agents from Patients with Inflammatory Bowel Disease. Gastroenterology 2015;149:1716–30.

147. van der Valk ME, Mangen M-JJM-JJ, Leenders M, Dijkstra G, van Bodegraven A a, Fidder HH, et al. Healthcare costs of inflammatory bowel disease have shifted from hospitalisation and surgery towards anti-TNF therapy: results from the COIN study. Gut 2014;63:72–9.

148. Rutgeerts P, D'Haens G, Targan S, Vasiliauskas E, Hanauer SB, Present DH, et al. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology 1999; Epub ahead of print 1999. Available from doi:10.1016/S0016-5085(99)70332-X.

149. Assa A, Matar M, Turner D, Broide E, Weiss B, Ledder O, et al. Proactive Monitoring of Adalimumab Trough Concentration Associated With Increased Clinical Remission in Children With Crohn's Disease Compared With Reactive Monitoring. Gastroenterology 2019;157:985-996.e2.

150. Roblin X, Williet N, Boschetti G, Phelip JM, Del Tedesco E, Berger AE, et al.

Addition of azathioprine to the switch of anti-TNF in patients with IBD in clinical relapse with undetectable anti-TNF trough levels and antidrug antibodies: A prospective randomised trial. Gut 2020; Epub ahead of print Jan 24, 2020. Available from doi:10.1136/gutjnl-2019-319758.

151. Lamb CA, Kennedy NA, Raine T, Hendy PA, Smith PJ, Limdi JK, et al. British Society of Gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults. Gut 2019;68:s1–106.

152. Torres J, Bonovas S, Doherty G, Kucharzik T, Gisbert JP, Raine T, et al. ECCO Guidelines on Therapeutics in Crohn's Disease: Medical Treatment. J Crohn's Colitis 2020;14:4–22.

153. Mehta P, Manson JJ. What Is the Clinical Relevance of TNF Inhibitor Immunogenicity in the Management of Patients With Rheumatoid Arthritis? Front Immunol 2020;11. Epub ahead of print Apr 7, 2020. Available from doi:10.3389/fimmu.2020.00589.

154. Knights D, Silverberg MS, Weersma RK, Gevers D, Dijkstra G, Huang H, et al. Complex host genetics influence the microbiome in inflammatory bowel disease. Genome Med 2014;1–11.

155. Tibble J, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S, et al. A simple method for assessing intestinal inflammation in Crohn's disease. Gut 2000;47:506–13.

156. Bonnín Tomàs A, Vila Vidal M, Rosell Camps A. [Fecal calprotectin as a biomarker to distinguish between organic and functional gastrointestinal disease]. Rev Esp Enferm Dig 2007;99:689–93.

157. Carroccio A, Iacono G, Cottone M, Di Prima L, Cartabellotta F, Cavataio F, et al. Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from irritable bowel syndrome: a prospective study in adults and children. Clin Chem 2003;49:861–7.

158. D'Incà R, Dal Pont E, Di Leo V, Ferronato A, Fries W, Vettorato MG, et al. Calprotectin and lactoferrin in the assessment of intestinal inflammation and organic disease. Int J Colorectal Dis 2007;22:429–37.

159. Otten CMT, Kok L, Witteman BJM, Baumgarten R, Kampman E, Moons KGM, et al. Diagnostic performance of rapid tests for detection of fecal calprotectin and lactoferrin and their ability to discriminate inflammatory from irritable bowel syndrome. Clin Chem Lab Med 2008;46:1275–80.

160. Schoepfer AM, Trummler M, Seeholzer P, Criblez DH, Seibold F. Accuracy of four fecal assays in the diagnosis of colitis. Dis Colon Rectum 2007;50:1697–706.

161. Colombel J-F, Panaccione R, Bossuyt P, Lukas M, Baert F, Vaňásek T, et al. Effect of tight control management on Crohn's disease (CALM): a multicentre, randomised, controlled phase 3 trial. Lancet (London, England) 2017; Epub ahead of print Oct 31, 2017. Available from doi:10.1016/S0140-6736(17)32641-7.

162. Peyrin-Biroulet L, Khosrotehrani K, Carrat F, Bouvier AM, Chevaux JB, Simon T, et al. Increased risk for nonmelanoma skin cancers in patients who receive thiopurines for inflammatory bowel disease. Gastroenterology 2011;141:1621–8.

163. Kotlyar DS, Lewis JD, Beaugerie L, Tierney A, Brensinger CM, Gisbert JP, et al.

Risk of Lymphoma in Patients With Inflammatory Bowel Disease Treated With Azathioprine and 6-Mercaptopurine: A Meta-analysis. Clin Gastroenterol Hepatol 2014;104–6.

164. Lichtenstein GR, Feagan BG, Cohen RD, Salzberg B a, Diamond RH, Chen DM, et al. Serious infections and mortality in association with therapies for Crohn's disease: TREAT registry. Clin Gastroenterol Hepatol 2006;4:621–30.

165. Pauwels RWM, van der Woude CJ, Nieboer D, Steyerberg EW, Casanova MJ, Gisbert JP, et al. P138 Prediction model to safely cease anti-TNF therapy in Crohn's disease: individual patient data meta-analysis (IPD-MA). J Crohn's Colitis 2019;13:S158–9.

166. Sazonovs A, Kennedy NA, Moutsianas L, Heap GA, Rice DL, Reppell M, et al. HLA-DQA1*05 Carriage Associated With Development of Anti-Drug Antibodies to Infliximab and Adalimumab in Patients With Crohn's Disease. Gastroenterology 2020;158:189–99.

167. Sazonovs A, Kennedy NA, Bewshea CM, Moutsianas L, Walker GJ, De Lange K, et al. 590 - HLA-DQA1 Contributes to the Development of Antibodies to Anti-TNF Therapy in Crohn's Disease. Gastroenterology 2018;154:S-124-S-125.

168. Sazonovs A, Kennedy NA, Bewshea C, Moutsianas L, Walker GJ, De Lange K, et al. Hla-dqa1 contributes to the development of antibodies to anti-TNF therapy in crohn's disease. Gut 2018;67:A52–3.

169. Sazonovs A, Kennedy NA, Bewshea C, Moutsianas L, Walker GJ, De Lange K, et al. HLA-DQA1 contributes to the development of antibodies to anti-TNF therapy in Crohn's disease. J Crohn's Colitis 2018;12:S009-S010.